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T -Cell Cytokines and Equine Nematode Infections.

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DEDICATION

For my parents, Ed and Nancy Johnson
Through love and support they have taught me the
importance of a positive attitude, hard-work and dedication.

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ABSTRACT

To address the role of parasites on subsequent antigenic challenge, humoral, lymphoproliferative, and cytokine responses to a single intramuscular injection of keyhole limpet haemocyanin (KLH) were compared between groups of ponies with high, medium or low gastrointestinal parasite burdens. Heavily and moderately parasitised animals showed a trend towards reduced KLH-specific lymphoproliferation and reduced antigen specific total immunoglobulin, IgG(T) and IgA compared to lightly parasitised ponies. The peripheral blood mononuclear cells (PBMC) from medium and heavily parasitised ponies also had significantly lower levels of IL-4. These data indicate that heavily parasitised animals have uniformly decreased cellular and humoral immune responses to soluble protein immunization.

The well-defined *Strongylus vulgaris* helminth naïve pony model was employed to investigate the cross-regulation of T-helper (Th1) cytokines on the induction of Th2 cytokines and to demonstrate the importance of Th2 responses in protective immunity to *S. vulgaris*. Whereas immunization of naïve ponies with radiation-attenuated third stage larvae (IrrL3) induces Th2 cytokines and is protective against *S. vulgaris* infection, soluble adult worm antigen (SAWA) in Ribi adjuvant induces Th1 cytokines and is not protective. Cytokine, humoral, and cellular responses were characterized to determine whether prior immunization with Ribi-SAWA would alter the IrrL3 induced protective immune response to *S. vulgaris*. Similar to ponies vaccinated only with IrrL3, animals immunized with both Ribi-SAWA and IrrL3 were still protected against challenge even though they demonstrated alterations in the protective immune response. Protection against *S. vulgaris* was associated with increased mRNA levels of IL-5 and

IL-13 within the cecal lymph node cells (CLNC) and increased blood and cecal eosinophil counts. Vaccination with Ribi-SAWA reduced the level of IL-5 in the CLNC and lowered the blood eosinophil count, but failed to alter the protective immune response. Elevated IL-4 and cecal mast cells did not correlate with protection against *S. vulgaris*. Antibody analysis revealed increased IgG(T), IgGa and IgA responses to L4 and L3 soluble *S. vulgaris* antigens in protected animals, along with an early IgGa, IgGb and IgG(T) antibody response to the surface of both early and developing L3.

INTRODUCTION

Nematode parasites are common in horses even in the era of highly effective broad-spectrum antiparasiticides. Often these parasites are able to persist within an infected host due to adaptations that enable them to avoid immune detection, recognition and clearance (reviewed by Riffkin et al., 1996; Maizels et al., 1993). Beyond simple avoidance strategies, nematodes also secrete molecules that modify the normal regulation of the host immune response (Grencis and Entwistle, 1997) and consequently may alter the host's ability to respond to additional antigenic stimuli. Widespread anthelmintic resistance and the lack of proper parasite monitoring have resulted in the persistence of nematodes even on farms where anthelmintic usage is incorporated into the management program. Therefore, an alternative to anthelmintic treatment, such as vaccination, is an attractive option. Development of effective vaccines, however, requires gaining a more precise definition of the protective equine immune response to nematode parasites. In addition, it is also necessary to identify inappropriate immune responses that may contribute to the pathologic conditions often associated with parasitism, such as diarrhea and colic. Understanding the mechanisms by which protective immune responses are generated and regulated is important for developing effective vaccine strategies as well as for investigating basic equine immunology and the consequences of nematode infection.

The following series of chapters addresses the characterization and regulation of the equine immune response to nematode parasites. These studies address the regulation of immunity to heterologous protein administration as well as to a specific parasite infection. The effects of a mixed gastrointestinal parasite infection on

subsequent antigenic challenge are initially described, followed by experiments that focus specifically on regulation of immunity to the nematode, *Strongylus vulgaris.* This parasite has a well-defined natural host-parasite system and has proven very useful in experimental studies of equine immunity to nematodes.

Chapter 2 reports on the effect of gastrointestinal parasites on a single intramuscular injection of keyhole limpet haemocyanin (KLH). The hypothesis was that immunity to heterologous vaccination would be improved after parasitised ponies were treated with an effective anthelmintic. Furthermore, it was expected that the response of parasitised ponies to KLH immunization would be polarized toward a T helper type 2 (Th2) cytokine profile. Humoral, lymphoproliferative, and cytokine responses to KLH were compared between groups of ponies with high, medium or low gastrointestinal parasite burdens. This experiment provides useful data regarding the effects of gastrointestinal parasites on subsequent antigenic challenge.

The potential cross regulation of Th2 cytokine induction by Th1 cytokines is reported in Chapter 3 using the *S. vulgaris*-helminth naïve pony model. The hypothesis tested was that in a milieu of Th1 cytokines, vaccination with radiation-attenuated *S. vulgaris* L3 (IrrL3) would not induce a dominant Th2 cytokine response that would render ponies susceptible to *S. vulgaris* challenge. In addition to measuring the local and peripheral cytokine and cellular immune responses, several clinical parameters were analyzed along with histologic examination of the ileo-cecal-colic artery, cecum and liver. These experiments further characterize the protective cellular immune response to *S. vulgaris* as well address the induction and regulation of these responses.

In Chapter 4, the effects of different immunization regimes on peripheral antibody responses and their relationship to protection were analyzed and discussed. Total IgG, IgG subisotype and IgA antibody levels to soluble adult worm antigen (SAWA), L4 and L3 antigens were measured using the ELISA technique. In addition, total IgG and IgG subisotype antibody titers that were directed to the surface of both early and developing *S. vulgaris* L3 were compared using the indirect fluorescent antibody technique (IFAT). The hypothesis was that protected animals would produce a distinct antibody profile to soluble adult, L4 and L3 antigens and to early and developing L3 surface antigens. The data generated from these experiments further characterize the protective IgG antibody response to *S. vulgaris* and provide evidence for cytokine regulation of antibody production in equids. The results of this work will help to better define the effect of gastrointestinal nematode parasitism on equine immune responses.

CHAPTER 1

LITERATURE REVIEW

Th2 Immunity in Helminth Infections

Understanding the polarized pattern of immune responses induced by helminth and protozoal parasites came with the discovery that infection with different pathogens resulted in the development of distinct $CD4^+$ T-cell subsets. $CD4^+$ T-cell clones from inbred mice were divided into 2 phenotypes based on their production of characteristic cytokine profiles. The T helper type 1 (Th1) cells produced interleukin-2 (IL-2) and INF-γ, and the Th2 cells produced IL-4 (Mosmann et al.1986; 1989). Since these initial experiments, it has been demonstrated that mature murine Th1 cells secrete IL-2, INF-γ, and lymphotoxin-alpha (LT-α), while Th2 cells secrete IL-4, IL-5, IL-9, IL-10, and IL-13 (Seder and Paul, 1994, reviewed in Spellberg and Edwards 2001).

Th1 and Th2 T-cell clones have different functions that are attributable to the different lymphokines they produce. For example, Th1 cytokines provide protection against a wide variety of intracellular pathogens, including parasites, bacteria and viruses through the stimulation of phagocytosis and macrophage activation (Daugelat and Kaufmann, 1996). Th2 cytokines, particularly IL-4, IL-5, and IL-13 protect against extracellular pathogens through activation of B-cell proliferation, antibody production, and eosinophilpoesis (reviewed in Spellburg and Edwards 2001). It has also been shown that specific cytokines from Th1 and Th2 cells, specifically INF-γ and IL-4, respectively, are potent cross-inhibitors of the two T-cell phenotypes (Seder and Paul, 1994).

The study of parasite-directed immune responses has provided functional evidence for the foundation of Th1 and Th2 responses. The first direct demonstration for the relevance of the Th1/Th2 paradigm was made in studies on the *Leishmania major* murine model. While most inbred strains of mice develop self-healing infections, BALB/c mice develop a non-healing and fatal infection accompanied by a hyper-responsive T-cell reaction. A series of experiments indicated that $CD4^+$ T-cells had the potential to mediate both susceptibility and resistance in mice infected with *L. major* (Reiner and Locksley, 1995). This suggested that differential stimulation of Th1 and Th2 cells could account for resistance and susceptibility (reviewed in Scott et al. 1989). Subsequently, it was reported that genetically resistant C57BL/6 mice produced high levels of INF-γ and little IL-4, while susceptible BALB/c mice produced high IL-4 and low INF-γ (Heinzel et al. 1989). Moreover, resistant mice could be made susceptible by administering anti-INF-γ or anti-IL-12 antibodies and susceptible mice could be made resistant by treatment with anti-IL-4 or IL-12 (reviewed in Scott et al., 1989; Reiner and Locksley, 1995). Additional studies on a wide range of parasites have supported the notion that Th1 responses are protective against intracellular protozoa. These studies have been performed with the parasites *Toxoplasma gondii* (Suzuki et al., 1989), *Cryptosporidium parvum* (Culshaw et al., 1997; Urban et al., 1996a), and *Plasmodium spp* (Sedegah et al., 1994). Although the general principals of the Th1/Th2 dichotomy are accepted, several exceptions have been reported. For example both Th1 and Th2 immune mechanisms operate against different life cycle stages of *Plasmodium spp.* since these parasites have both extracellular (free sporozoites and merozoites in the

blood) and intracellular stages (intra-hepatocyte and intra-erythrocytic (reviewed in Doolan and Hoffman, 1997).

In contrast, studies with the helminth parasite *Trichuris muris* provided early evidence that protective Th2 responses were elicited toward extracellular parasites. For example, AKR mice were susceptible to *T. muris* and developed long-term infections, while BALB/k mice rejected the parasites shortly after exposure (Wakelin 1975; Else et al., 1992). In this system, resistant mice expressed Th2 responses, specifically IL-4 and IL-13, and susceptible mice produced the Th1 cytokines IL-12 and INF-γ. Furthermore, IL-12 administration induced a switch from a protective Th2 to an infection-permissive Th1 response (Else et al., 1994; Bancroft et al., 1997).

While immune expulsion of gastrointestinal (GI) helminth parasites is usually associated with Th2 responses, the effector mechanisms directly responsible for parasite elimination have not been elucidated. In experimental animal models, the pathology associated with GI parasite infection is characterized by villous atrophy, crypt hyperplasia, goblet cell hyperplasia, and infiltration of the mucosa by a variety of inflammatory cells of which eosinophils and mast cells predominate (reviewed by Garside et al., 2000). While protective responses have been associated with Th2 cytokines, including IL-3, IL-4, IL-5, IL-9 and IL-13, as well as IgE and IgG1 antibody responses, Th1-mediated events appear antagonistic in terms of protection (Else et al., 1994).

Given the diverse lifecycles of GI helminths, there seems to be no single immunological mechanism capable of conferring host protection. However, many studies with rodent models have shown that IL-4 is the key Th2 cytokine that promotes

induction of immunity against GI nematode parasites. Activated $CD4^+$ T-cells are the primary producers of IL-4 (Paul, 1991). However, IL-4 is also produced by a variety of cells known as $CD4^+NK1.1^+$ natural T-cells, mast cells, and basophils (Leonard 1999). IL-4 acts as a B- and T-cell growth factor and is vital for immunoglobulin class switching to IgE and IgG1 in mice, and IgE and IgG4 in humans. It also augments expression of class II major histocompatibility complex (MHC) molecules and IgE receptors on B-cells (Leonard 1999). IL-4 stimulates mucosal mastocytosis (Madden et al., 1991), promotes Th2 cytokine responses (Spellburg and Edwards 2000) and enhances the expression of vascular cell adhesion molecule-1 (VCAM-1) (Schleimer et al., 1992). In addition, IL-4 stimulates production and eosinophil responsiveness to the β chemokine, eotaxin (Li et al., 1999). It also suppresses production of inflammatory mediators such as prostaglandins, reactive nitrogen and oxygen intermediates, as well as the Th1 cytokines IL-12 and TNF-α (reviewed by Finkelman et al., 1999).

The mechanism by which IL-4 specifically mediates protection against GI helminths is unknown. In uninfected mice, IL-4 administration has dramatic effects on intestinal physiology through a reduction in peristalsis, increased permeability, and decreased fluid absorption. These responses appear to be T-cell and mast cell dependent and their net effect is to increase the intestinal fluid content. Although not proven, an IL-4 dependent increase in intestinal fluid content may aid in the elimination of intestinal parasites (Leonard et al., 1999). The role of IL-4 in helminth infections has been demonstrated in mice infected with *Heligomosomoides polygyrus* (Urban et al., 1991), *Trichuris muris* (Else et al., 1994), and *Trichinella spiralis* (Finkelman et al., 1997). In each parasite system, IL-4 plays a unique role in mediating protection either

through the induction of a Th2 cytokine response, mucosal mastocytosis, and/or parasite specific IgE and IgG antibodies.

The Th2 cytokine IL-13 shares many biologic functions with IL-4. IL-13 mimics the functions of IL-4 by binding to the α-chain of the IL-4 receptor (reviewed by Finkelman et al., 1999) and signaling through a shared signal transducer and activator of transcription (STAT) 6-dependent pathway (reviewed by McKenzie 2000). Since there is a difference in relative expression of receptor chains on different cell types, some cells are more responsive to IL-4 or IL-13. For example, murine B-cells as well as human and murine T-cells are more responsive to IL-4 (Zurawski. and de Vries 1994), while human smooth muscle cells and epithelial cells are more responsive to IL-13 (Finkelman et al., 1999). There are also differences in the production and kinetics of IL-4 and IL-13. While T-cells, mast cells and basophils produce both cytokines, IL-13 is also produced by dendritic cells and natural killer cells. Also, in mice, only Th2 cells secrete IL-13, whereas in humans both Th1 and Th2 cells produce IL-13 (Finkelman et al., 1999; Zurawski and de Vries, 1994). Also, IL-4 responses diminish during the course on an immune response whereas IL-13 responses are sustained. (Finkelman et al., 1999; Zurawski and de Vries, 1994).

Since IL-13 levels are sustained throughout an infection, it has been hypothesized that IL-13 may play a more substantial role during elimination of chronic helminth infections. In addition, goblet cell hyperplasia is a common sequela of chronic parasitic infections and appears to be IL-13 related. In immunity to GI helminth infections, mucin proteins produced by goblet cells may play an important role in

parasite expulsion by enveloping the parasites and/or interrupting adhesion and feeding (Garside et al., 2000).

IL-13 has also been shown to be a key cytokine in mediating protection against the GI parasites *Nippostrongylus brasiliensis* and *T. muris*. For example, IL-4 knockout ($^{-/-}$) mice expel *N. brasiliensis* normally from the gut, whereas IL-13$^{-/-}$ and IL-4/IL-13 double$^{-/-}$ mice are extremely susceptible to *N. brasiliensis* infection (Urban et al., 1998; McKenzie et al, 1999). In addition, IL-13$^{-/-}$ mice are unable to expel a primary *T. muris* infection even in the presence of the Th2 cytokines IL-4, IL-5, and IL-9, parasite specific IgG1 and IgE, and a cecal mastocytosis (Bancroft et al., 1998).

Similarly to IL-4 and IL-13, a correlation between the Th2 cytokine IL-5 and GI nematodes has also been established. IL-5 promotes the growth and differentiation of human and murine eosinophils, and induces the release of eosinophils from the bone marrow (reviewed in Finkelman et al 1991). IL-5 also heightens the killing activity of eosinophils and enhances murine B-1 B lymphocyte function and IgA production (Leonard 1999). Prominent sources of IL-5 include T-cells, mast cells, eosinophils, and NK cells (Lalani et al., 1999).

Although helminth infections are correlated with an increase in serum IL-5, eosinophilia, and eosinophil infiltration around invading parasites, a direct role for IL-5 in parasite killing is still unclear. IL-5 deficient mice do not develop blood or tissue eosinophilia when infected with parasites, but the subsequent effect on parasite burden varies with the specific parasite studied. For example, there is no correlation between IL-5 levels and the survival, growth, and fecundity of *Toxocara canis* (Sugane et al., 1996), *T. muris* (Betts and Else, 1999), *H. polygyrus*, or *N. brasiliensis* (Leonard et al.,

1999. However, a role for IL-5 has been established in protective immunity against the nematodes, *Strongyloides stercoralis* (De'Broski et al, 2000), *S. venezuelensis* (Korenaga et al., 1991), *S. ratti* (Matthaei et al., 1997) and *Angiostrongylus cantonensis* (Sasaki, et al., 1993). Mice infected with these parasites and treated with an anti-IL-5 antibody have impaired parasite clearance. Conflicting results exist for the role of eosinophils in protection against *T. spiralis* challenge infections. Herndon and Kayes (1992) reported that depletion of eosinophils in mice following treatment with anti-IL5 antibody did not alter *T. spiralis* parasite burdens following primary and challenge infections. However, depletion of eosinophils through disruption in the IL-5 gene was shown to increase parasite burdens and delay worm expulsion one week following administration of a challenge infection but not a primary infection. (Vallance et al., 2000). Moreover, intestinal hypercontractility was moderately impaired in *T. spiralis* infected mice suggesting that there may be an additional role for IL-5 in the regulation of enteric muscle function during inflammation (Vallence et al., 1999).

Th2 Immunity in Gastrointestinal Nematode Infections

Nematodes occupy a variety of niches within the intestine including: lumenal (*N. brasiliensis)*, tissue penetrating (*H. polygyrus*), and intraepithelial (*T. spiralis* and *T. muris*). The pathology associated with each varies according to the particular host-parasite combination. *N. brasiliensis*, a trichostrongylid nematode of the rat, has been adapted to the mouse for experimental purposes. Mouse-adapted stains of *N. brasiliensis* penetrate the host skin and migrate to the lungs within 24-48 hours. Larvae are coughed up and swallowed and mature in the jejunum by 5-6 days after infection (Finkelman et al., 1997). A series of experiments have shown that both IL-4 and IL-13

contribute to the expulsion of *N. brasiliensis*. Both cytokines operate through an IL-4 receptor alpha-dependent, STAT6-dependent mechanism, with IL-13 playing the major role in expulsion. However, a role for IL-4 has been delineated in SCID and anti-$CD4^+$ treated mice. In these mice, IL-4 administration was sufficient to cause expulsion during chronic infections (reviewed by Else and Finkelman, 1998). A role for IL-4, however, has been shown to be strictly redundant since anti-IL-4 mAb-treated mice are as resistant to infection as the wild type mice (Finkelman et al., 1991). Infections with *N. brasiliensis* are accompanied by an increase in intestinal goblet cell numbers, increased mucus production, and a change in mucus carbohydrate content (Nawa et al., 1994; Finkelman et al., 1997). These changes in mucus production are T-cell-dependent and have been hypothesized to favor parasite expulsion. Increased mucus production may function to immobilize parasites and/or inhibit intestinal adhesion (Finkelman et al., 1997).

Parasite-directed antibodies, mast cells and Th1 cytokines are not associated with protection to *N. brasiliensis*. In fact, susceptible STAT $6^{-/-}$ mice induce a strong anti-parasite IgG1 and IgG2 antibody response as well as a profound intestinal mastocytosis which do not aid in the elimination of worms (Urban et al., 1998b). As with other GI nematodes, several studies have shown that Th1 cytokines are not protective in *N. brasiliensis* infections. Treatment of *N. brasiliensis*-infected mice with INF-γ or IL-12 at the start of a primary infection enhances egg production and prolongs the course of infection. Also, if IL-12 treatment is given throughout the course of a challenge infection, worm expulsion is suppressed (Finkelman, et al., 1997).

Heligomosimoides polygyrus is a nematode parasite of the mouse that establishes chronic infections. The infective third stage larvae are ingested and enter the mucosa of the anterior small intestine. Immature larvae reside in the muscularis externa for eight days prior to emerging into the intestinal lumen. Once in the lumen, larvae mature to adults and feed on host intestinal mucosa for several months before being expelled. Although primary infections are chronic, challenge infections are quickly expelled (Finkelman et al., 1997). A clear role has been established for IL-4 induced protection against *H. polygyrus*. The ability of mice to expel a challenge infection can be blocked for 17 days by administration of mAbs against IL-4 or the IL-4 receptor (reviewed by Else and Finkelman, 1998). In addition, IL-4 $^{-/-}$ mice are unable to mount a protective response to challenge infections (Finkelman et al., 1997). Likewise, administration of IL-4 decreases egg production and induces worm expulsion during a primary infection. Investigators have also demonstrated a protective role for humoral immunity during *H. polygyrus* infections. Studies have shown that administration of large volumes of immune serum transfers protection against *H. polygyrus*, and that parasite directed IgG1 is the principle protective isotype (Dobson, 1982). There may also be a role for mast cells in immunity to *H. polygyrus* where resistant mouse strains exhibit an intestinal mastocytosis during worm expulsion (Else and Finkelman 1998).

Trichuris muris is nematode of mice that following ingestion establishes residency within the cecum and large intestine. The anterior end of the worm embeds into and digests host mucosal epithelium. Chronic infections occur in some mouse strains, but it is expelled from other strains before egg-laying adults can develop

(Finkelman et al., 1997). Many studies have demonstrated a clear role for IL-4 in protection to *T. muris*. Immunity to *T. muris* is abrogated in IL-4 $^{-/-}$ mice and in mice administered mAbs against the IL-4 receptor. Furthermore, when IL-4 is given in vivo, it facilitates expulsion of a primary *T. muris* infection. IL-13 is also shown to be important in resistance to *T. muris* and is produced in large amounts during the generation of a protective immune response. Mice with a targeted disruption in the IL-13 gene are fully susceptible to infection in the face of an otherwise normal Th2 response (Spellburg and Edwards, 2000; Else and Finkelman, 1998). These data suggest that IL-4 is required to initiate the protective Th2 response, however, IL-13 signaling through the IL-4-receptor-alpha is necessary for parasite clearance.

The deleterious effects of Th1 cytokines have been clearly demonstrated in *T. muris* susceptible mice. Susceptible mice respond to primary infections by producing high levels of INF-γ and parasite specific IgG2a. These mice develop long term chronic infections and are unable to expel the parasites (Else et al., 1994). However, if these mice are treated with anti-INF-γ or anti-IL-12 mAbs at the beginning of an infection, susceptible mice are able to expel the parasite and mount a Th2 response. Conversely, treatment of resistant murine strains with IL-12 allows establishment of a chronic infection through the induction of INF-γ (Else et al., 1994; Else and Finkelman 1998).

Trichinella spiralis is another GI dwelling nematode in mice that has been intensely studied. The first stage larvae are encysted in muscle tissue and when ingested by a susceptible host they ecdyse, enter the duodenal or jejunal epithelium, and mature into adults. The adults induce syncytial cell formation and reside within the intestinal epithelial cells. Female worms release newborn larvae that are picked up by

venous blood and carried throughout the body eventually settling into striated muscles (Finkelman et al., 1997).

A primary role for IL-4 and possibly IL-13 has been shown in the development of resistance to primary infections with *T. spiralis*. Mice treated with the anti-IL-4-receptor mAb exhibit prolonged adult infections and harbor elevated muscle larval burdens. IL-9 is another Th2 cytokine that is important for mast cell development and appears to have a protective role against *T. spiralis*. Resistant mice produce high levels of IL-9 that correlate with a prominent intestinal mastocytosis (Else and Finkelman, 1998). Mast cells have been shown to contribute to intestinal inflammation through the production of proteinases, cytokines and inflammatory mediators (Garside et al., 2000; Miller, 1996). The use of anti-stem cell factor or *c-kit* antibodies, which severely depress intestinal mastocytosis, showed that a mast cell deficiency inhibited the expulsion of a primary *T. spiralis* infection. Furthermore, when IL-9 was expressed at high levels in transgenic mice, there was a heightened Th2 cytokine response, elevated mastocytosis and rapid worm expulsion (Else and Finkelman, 1998). It was hypothesized that the exacerbated intestinal mastocytosis lead to high IL-4 release resulting in escalation of the Th2 response (Leonard et al., 1999).

IgE is the classic mast cell activator and evidence from studies with *T. spiralis* in the rat implicates an important function for IgE in mediating protection. Rapid expulsion can be transferred with purified IgE antibody while suppression of the IgE response results in elevated muscle larvae burdens (Ahmad et al., 1984; Else and Finkelman, 1998). The exact mechanism by which IgE mediates resistance to *T. spiralis* is not known. Several cell types are known to bear IgE Fc receptors including

mast cells, eosinophils, macrophages, monocytes and platelets likely cooperate with IgE in antibody dependent cell mediated cytotoxicity (ADCC) reactions (Leonard et al., 1999). Specific evidence for ADCC killing has been shown in vitro with *Brugia pahangi* and *S. mansoni* (reviewed by Lobos, 1997).

Beyond humans and rodent models, there is no definitive proof for the existence of Th1/Th2 T-cell subsets in other animal species. There is evidence, however, that additional animal species are capable of mounting immune responses consistent with a Th2 cytokine response. In ruminants, there are many features of the immune response to nematode infections that correlate with Th2 induced immunity. For example, eosinophilia and mucosal mast cell hyperplasia are often observed during GI helminth infections in sheep and cattle (reviewed by Claerebout and Vercruysse, 2000). Unlike in rodents, the role of specific cytokines in regulating these responses against GI nematodes has yet to be determined. However, it has been demonstrated that prolonged administration of anti-INF-γ Mab results in significantly reduced fecal egg counts and total worm burdens in sheep following a primary infection with *Trichostrongylus colubriformis* (McClure et al., 1995). The expression of cytokine mRNA in *T. colubriformis*-resistant and –susceptible lambs shows that mesenteric lymph node cells from both lines express high levels of IL-2, IL-4, and INF-γ. However, IL-4 expression is increased in resistant lambs following a three-day culture with *T. colubriformis* excretory-secretory antigen (Pernthaner et al., 1997).

Data also suggest that resistance to the ovine nematode, *Haemonchus contortus*, is a Th2 mediated event. Resistant lambs produce elevated IL-5 and reduced INF-γ following antigen- and mitogen-induced stimulation. Likewise, parasite-specific IgG1

and IgE antibodies are elevated in the resistant lambs while histologic examination reveals higher densities of mast cells and eosinophils within the abomasal mucosa (Gill et al., 2000).

In cattle, cytokine profiles during a primary *O. ostertagi* infection show a less restricted Th1/Th2 profile. Naïve animals produce increased IL-4, IL-10, and INF-γ within the abomasal and mucosal lymphocytes following a primary infection. There are also reduced percentages in the number of T-cells and increased percentages in the number of B-cells following challenge (Canals et al., 1997). In contrast, protected cattle showed a decreased IL-4 response following challenge. Protected animals also show a positive correlation between the diminished IL-4 mRNA levels and the number of worms recovered at necropsy. These results suggest that the Th2 cytokine, IL-4, may not be associated with protective immunity to *O. ostertagi* (Almeria, et al., 1998). This interpretation is in conflict with the results from murine studies suggesting Th2 cytokines are required for protective immunity to GI helminths. However, neither bovine T-cell clones nor in vivo T-cell responses demonstrate a clear Th1/Th2 cytokine expression pattern as reported in murine and human T-cell clones (Almeria et al., 1997, 1998; Canals et al., 1997).

Th2 Immunity in Tissue Migrating Nematode Infections

Many helminths occupy niches within the host that are outside of the GI tract. Some species are totally confined to the parental tissues while others undergo development in the tissues prior to establishing within the intestine. The close contact between tissue-dwelling worms and the host immune system results in direct access of parasite antigens to host effector agents such as antibody, complement, and eosinophils.

Eosinophils have historically been recognized as a distinctive feature of the immune response elicited toward helminth infections (Behm and Ovington, 2000). The demonstration that eosinophils could kill the early schistosomula stage of *Schistosoma mansoni* in vitro sparked research into their role as major effector cells in resistance to helminth infections (Meeusen and Balic, 2000). Eosinophil-mediated killing has been shown to be most effective against tissue-migrating larval stages and requires cooperation with antibody and/or complement. In vitro, eosinophils adhere to immunoglobulin coated larvae resulting in granule release. In vivo, degranulated eosinophils aggregate in the locality of helminthes and eosinophil-derived proteins can be found on the surfaces of dead and damaged larvae (Behm and Ovington 2000; Pritchard et al., 1997). Eosinophil proteins that appear to be directly associated with larval damage include eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and major basic protein (MBP). Worm tegument has been shown to be highly anionic and, therefore, it is susceptible to damage by these intensely basic proteins (Pritchard et al., 1985). Both antibodies and eosinophils have been suggested to play a role in protection against tissue migrating helminths such as *Strongyloides spp.* (Grove et al., 1986; Korenaga, et al., 1991), *A. cantonensis* (Sasaki et al., 1993), and *H. contortus* (Rainbird et al., 1998).

Strongyloides stercoralis is a parasitic nematode of humans and dogs that has been adapted to the murine model. In the mouse, third-stage larvae migrate through muscle tissue up to 9 days following primary infection, but are cleared prior to developing to the adult stage. Parasite clearance is associated with an eosinophilic infiltrate that surrounds the dying larvae within the muscle tissue (Grove et al., 1986).

Since adult worms develop within SCID mice, it is hypothesized that L3 development in other mice strains is prevented by the host immune response (Rotman et al., 1995). Adaptive protective immunity against *S. stercoralis* larvae in BALB/cByJ mice was abrogated by treatment with either IL-12, anti-IL-5 or anti-IL-4 mAb (Rotman et al., 1997). Also, naïve IL-5 transgenic mice were shown to be resistant to infection and developed eosinophil infiltration with granule release into the area of larval migration. $CD4^+$ T-cell restricted IL-5 production and elevated parasite specific IgM production characterized adaptive immunity in wild-type mice. Also, administration of parasite-specific IgM or transfer of eosinophils to IL-5 $^{-/-}$ mice could transfer protective immunity (De'Broski, et al., 2000).

Similarly to *S. stercoralis*, IL-5 has also been shown to play a protective role in immunity against the closely related rodent parasite *Strongyloides venezuelensis*. Treatment with anti-IL-5 or anti-IL-5 receptor Mab resulted in a suppression of peripheral and tissue eosinophilia and impaired resistance to migrating larvae. However, depletion of IL-5 and eosinophilia did not have an effect on primary or secondary immunity to the intestinal phase of the infection (Korenaga, et al., 1991).

Angiostrongylus cantonensis, a lungworm in the rat, causes eosinophilic meningitis in non-permissive hosts such as the mouse. During infection with *A. cantonensis*, mice evoke systemic and cerebrospinal (CSF) eosinophilia that is associated with the reduction in intracranial worms (Sugaya and Yoshimura, 1988). It has also been shown that rat eosinophils attach to and kill adult worms by an IgG dependent mechanism (Yoshimura et al., 1983). Furthermore, IL-5 depleted mice

infected with *A. cantonensis* show a depression of CSF eosinophilia and an enhanced survival of intracranial worms (Sasaki et al., 1993).

The role of eosinophils in protection against the tissue-invasive L3 stage of *Haemonchus contortus* has been suggested. Eosinophils were obtained from sheep primed by repeated infections with *H. contortus*. The primed eosinophils immobilized *H. contortus* larvae by an antibody and compliment dependent in vitro mechanism. Likewise, primed eosinophils incubated with larvae resulted in eosinophil degranulation and adherence to the larval surface. (Rainbird et al., 1998). These data suggest that eosinophil-mediated killing may be an effective mechanism against several species of tissue-dwelling parasites.

Th2 Immunumodulation of Heterologous Antigens

Eosinophils, mast cells, and parasite-directed antibodies are effector mechanisms that helminth parasites evoke through the induction of Th2 cytokines (reviewed in Else and Finkelman, 1998). There are several examples of the polarizing Th2 immune response induced by helminths modulating the Th1 mediated immune responses directed towards bacterial and viral pathogens. The filarial parasites *Brugia malayi* and *Onchocerca volvulus* skew the classic Th1 immune response to *Mycobacterium tuberculosis* towards a mixed Th1/Th2 profile (Stewart et al., 1999; Pearlman et al., 1993). Mice co-infected with the trematode *Fasciola hepatica* and the bacteria *Bordetella pertussis* exhibit a diminished Th1 response to *B. pertussis* that is reversed in IL-$4^{-/-}$ mice (Brady et al., 1999). Likewise pigs infected with *Trichuris suis* show a predilection toward bacterial-induced mucohemorrhagic enteritis (Mansfield and Urban, 1996). Immunomodulation to viral infections has also been demonstrated in

mice coinfected with *Toxocara canis* and Japanese encephalitis virus where co-infected mice demonstrated clinical encephalitis along with reduced viral specific antibody production and impaired T-cell responses compared to mice infected only with the virus (Gupta and Pavri, 1987). Similarly, mice co-infected with *Schistosoma mansoni* and vaccinia virus have reduced $CD8^+$ cytotoxic T-cell responses, reduced INF-γ production and delayed viral clearance (Actor et al., 1993). As demonstrated by these examples helminth parasites have been shown to not only modulate a host's response but also to suppress immune responses. Cattle infected with *O. ostertagi* display a profound, but transient nonspecific immunosuppression to mitogen and antigen induced stimulation (reviewed by Gasbarre 1997). Likewise, mice infected with *N. brasiliensis* show a depressed humoral immune response when vaccinated with sheep red blood cells that directly parallels the number of adult worms present in the small intestine (Crawford et al., 1989).

Parasitised animals exhibiting a dominant Th2 cytokine profile are also shown to produce a biased Th2 response to heterologous protein immunization. For instance, mice infected with *Schistosoma mansoni* produce increased IL-4 levels and diminished INF-γ and IL-2 levels following vaccination with sperm whale myoglobin (SwMb). In contrast, uninfected mice that were vaccinated with SwMb produced high levels of INF-γ and IL-2 and low IL-4 (Kullberg, et al., 1992). In a similar study, *N. brasiliensis* excretory/secretory proteins were co-administered with hen-egg lysozyme (HEL). These mice produced HEL-specific lymphoproliferative responses, IL-4 release, and IgG1 antibody production (Holland et al., 2000). These studies illustrate the potential for helminth antigens to act as Th2 adjuvants for unrelated antigens.

Immunity to *Strongylus vulgaris*

Strongylus vulgaris is a highly pathogenic GI nematode of equids. It undergoes extensive larval migration within the intestinal arteries prior to adult establishment in the cecum and large intestine. Infective third stage larvae (L3) are ingested with herbage from a contaminated pasture. The larvae exsheath as they initially pass through the stomach and small intestine and within two days penetrate the mucosa of the ileum, cecum, and ventral colon. By 4 to 7 days, L3 molt to the L4 stage and penetrate the submucosal arterioles. The L4 then migrate up the intestinal vasculature until reaching the cranial mesenteric artery 11 to 14 days post-infection. Larvae typically remain within the cranial mesenteric and ileo-cecal-colic arteries for an additional 120 days prior to the fifth stage (L5) molt. The L5 then travel via the blood to the small arteries on the serosal surface of the cecum and large intestine. Within the affected arteries, nodules form around the larvae and eventually rupture allowing *S. vulgaris* to emerge into the intestinal lumen. Following emergence, L5 require an additional 6 to 8 weeks to reach sexual maturity at which time the development of patency completes the 6-month lifecycle (Ogbourne and Duncan, 1985; McCraw and Slocombe, 1976).

Establishment of adult parasites in the cecum and large intestine results in chronic weight loss, diarrhea, and anemia. However, larval migration within the intestinal vasculature causes a more severe clinical disease (Morgan et al., 1991). Initial larval penetration of the ileum, cecum, and ventral colon creates multiple hemorrhagic foci and gives rise to a febrile response 48 hours post-challenge (Monahan et al., 1994).

Subsequent migration results in arteritis and thrombosis of the small submucosal arteries and clinically induces pyrexia, depression, anorexia, and abdominal pain all within the first 14 days of infection (Duncan and Pirie, 1972; Klei et al., 2000). Lesions within the ileo-cecal-colic and cranial mesenteric arteries are evident by 25 days post-infection and include subintimal larval tracts, thrombosis, fibrosis, and verminous aneurysms (White et al., 1985). In severe cases, large thrombi develop within the arterial lumen and release emboli that compromise perfusion to downstream intestinal vascular beds. The syndrome is termed verminous arteritis or thromboembolic disease and is characterized by ischemic infarction of the small and/or large intestine resulting in severe abdominal pain, toxemia, and death (White et al., 1985).

Prior to marketing of the avermectin class of anthelminthics, *S. vulgaris* was considered the most important equine parasite due to a 70-90% prevalence rate and the severe consequences imparted by verminous arteritis (Ogbourne and Duncan, 1985). Ivermectin, the first avermectin developed, kills both migrating and adult stages of *S. vulgaris* and regular treatment has dramatically reduced the rate of its infection and disease (Klei, 1992). Despite the reduced incidence, *S. vulgaris* still remains a cause of colic in areas where treatment for parasites is not provided and is an excellent research model for studying equine immunity to nematode parasites.

As a model, experimental challenge of parasite-free ponies with *S. vulgaris* provides several advantages. For example, infections of 500-1000 L3 are rarely fatal but still produce demonstrable clinical signs such as pyrexia, anorexia, depression, and mild colic within the first 2 weeks of infection. Also, monospecific infections are readily established by surgical implantation of adult worms into the cecum of parasite-

free ponies (McClure et al., 1994). Fecal culture from these ponies provides a ready source of L3 that can be used for oral challenge, antigen preparation, in vitro immunologic assays, and larval culture. Methods for the in vitro culture of late L3 and L4 have also been developed and these stages are used for immunologic assays and antigen preparation (Chapman et al., 1994). Most importantly, an experimental oral vaccine using radiation-attenuated *S. vulgaris* L3 (IrrL3) has been developed. This vaccine significantly reduces larval burdens and prevents the classic lesions of verminous arteritis (Klei et al., 1982; Klei et al., 1986). The IrrL3-induced protection is species-specific and is effective against both natural and experimental challenge (Klei et al., 1986; 1989). Because IrrL3 vaccinated ponies are nearly 100% protected, the mechanisms associated with protective immunity can be studied and characterized. The majority of information known regarding equine immunity to nematode infections has been collected in studies using this model.

In horses, acquired resistance to *S. vulgaris* is incomplete and concominant in nature. Low numbers of larval parasites exist within the intestinal vasculature while newly acquired L3 are expelled. The mechanisms by which L4 and L5 stages evade an active immune response are unknown. It is hypothesized that changes in surface antigen structures may allow larvae to escape immune detection. Alternatively, larvae may adapt to or modulate the host immune response within their local environment.

Parasite-free ponies infected with *S. vulgaris* have elevated eosinophils 3 to 5 weeks following challenge (Duncan and Pirie, 1975). This primary eosinophilia drops after 6 to 8 weeks and is followed by a second peak 2 to 3 weeks later (Bailey, 1984). Corresponding with the peripheral eosinophilia, cecal eosinophils begin migrating into

the submucosa after the first week of infection and are sustained for 2 to 4 months. After approximately 4 months larvae return to the intestine and eosinophil levels begin to decline (Duncan and Pirie, 1972, 1975).

An anamnestic eosinophilia is associated with the development of acquired immunity in IrrL3 protected ponies 1- 2 weeks following challenge (Swiderski et al., 1998a; Monahan et al., 1994). Recent studies also demonstrate a significant elevation in intestinal eosinophil counts in IrrL3 protected ponies compared to nonimmune animals at 16 and 35 days post-infection (Horohov et al., unpublished data; Monahan et al., 1994). Furthermore, IrrL3 vaccinates have a predominately eosinophil infiltration of the cranial mesenteric arterial adventitia at day 35. In contrast, histologic examination of the ileo-cecal-colic artery from nonimmune ponies reveals a predominately lymphoplasmacytic rather than eosinophilic inflammatory cell infiltrate (Monahan et al., 1994). Eosinophils from the IrrL3 recipients also stain dark grayish green with a faint or indistinct outline of the cytoplasmic granules that is suggestive of activation (Monahan et al., 1994).

Antibody-dependent cell-mediated cytotoxicity of migrating larvae has also been hypothesized to be a major effector mechanism in a number of helminth infections. Activated eosinophils in the presence of immune serum attach to and kill *S. stercoralis* L3 as well as newborn larvae of *T. spiralis* (Nolan et al., 1995; Venturiello et al., 1995). Similarly, activated eosinophils from *S. vulgaris* infected ponies adhere to and immobilize L3 in the presence of immune serum or immunoglobulins (Klei et al., 1992). In vitro analysis has demonstrated that eosinophils and neutrophils from *S. vulgaris* infected ponies express more Fc and complement receptors than do eosinophils

and neutrophils from strongyle naïve ponies (Dennis et al., 1988). Moreover, the serum-mediated cellular response is stage- and species-specific. Immune serum does not induce adherence of buffy coat cells to *Strongylus edentatus* or cyathostome L3 (Klei et al., 1992) and specificity is lost after the molt to the L4 stage (Monahan et al., 1994).

To study the role of antibodies during *S. vulgaris* infection, 1.5 L of heat inactivated immune serum was adoptively transferred to parasite-free ponies (Klei et al., 1992). Ponies were administered immune serum prior to *S. vulgaris* challenge and during the first three days of infection. The adoptive transfer of immune serum was not protective, however, it did reduce the severity of intravascular thrombosis and significantly increased L3 surface-specific antibody titers. Immune serum also induced an anamnestic eosinophilia and a markedly exacerbated eosinophil perivascular inflammatory response (Klei et al., 1992).

These data demonstrate that antibodies alone are insufficient to provide protection to *S. vulgaris*, however, does not totally eliminate their role in protective immunity. Circulating β-globulins are elevated in the serum of infected equids and IgG(T) is the predominate isotype produced (Klei et al., 1983; Bailey et a., 1989; Patton et al., 1978). Recent studies have revealed that pre-challenge levels of *S. vulgaris*-specific IgG(T), IgGa, and IgGb are significantly higher in the sera of IrrL3 vaccinates compared to naïve animals (Swiderski et al., 1999a) and following challenge, IgG(T) levels remained significantly higher in the IrrL3 recipients (Swiderski, et al., 1999a). Challenge with *S. vulgaris* also results in significantly increased IgA levels in both IrrL3 vaccinated and nonvaccinated ponies while elevated IgGa levels are only seen in

the nonvaccinates (Swiderski et al., 1999a). The biological function and cytokine regulation of equine isotypes is not known, therefore, the significance of the different isotypic response between vaccinates and nonvaccinates is unknown. Coincidentally, intramuscular immunization with soluble adult or larval extracts with Ribi adjuvant (Ribi-SAWA) induces an anamnestic IgG response to both adult and larval antigens. The vaccination, however, is not protective and results in the exacerbation of lesions normally associated with larval migration (Monahan et al., 1994). The exacerbation of arterial lesions seen in the face of high antibody titers does not rule out a role for antibodies in protective immunity. Although antibodies to somatic antigens are produced in Ribi-SAWA vaccinates, these ponies do not elicit an IgG response against the surface of early or developing L3 stages. The lack of a specific IgG response to the infective L3 stage may attribute to the absence of a protective immune response. Lack of protection may also result from poor or inappropriate cellular immune mechanisms and/or an insufficient mucosal antibody responses.

Although the IgG isotypic response to L3 surface antigens has not been characterized, IrrL3-induced protection is associated with high IgG levels prior to and following challenge based on indirect fluorescent antibody titers (IFAT) (Klei et al., 1983; Monahan et al, 1994). Attempts to identify species-specific surface antigens of L3 by extensive absorption of sera with cross-reacting antigens identified two putative surface proteins of 52 kDa and 36 kDa. These proteins were also detected by L3 surface labeling with iodogen, however, immune serum failed to immunoprecipitate the antigens (Klei, 1992).

Western blot analysis of somatic antigens of late L4, L5, and adult *S. vulgaris* show that several protein antigens are specifically immunogenic in individuals that acquire protective resistance (Klei, 1992). Sera from ponies with monospecific infections of *S. vulgaris, S. edentatus,* or *Parascaris equorum,* demonstrate some species- and stage-specific protein bands. Sera from all *S. vulgaris* infected ponies but not those infected with other parasites recognizes two protein bands of 98 kDa and 84 kDa in extracts of the L4 and L5 stages. Serum from all *S. vulgaris* challenged ponies also recognize two species-specific bans of 22 kDa and 23 kDa in adult antigen preparations. These bands can not be detected in the sera from ponies prior to challenge. In addition, only serum from IrrL3 vaccinated ponies recognizes a 21-kDa band in adult worm extracts. The relevance of these adult and larval antigens to protective resistance is currently unknown, however, they may represent important immune targets, particularly in ADCC reactions.

Factors, which act as non-specific mitogens for non-sensitized lymphocytes, have been demonstrated in several parasite species including *Trypanosoma brucei* and *Taenia solium* (Selkirk et al., 1981; Sealy et al., 1981). Studies of naturally-infected nonimmune horses indicate that lymphoproliferative responses to *S. vulgaris* SAWA, L4, and L5 antigens are inversely related to larval burdens with helminth-free ponies being more responsive to stimulation. Animals exposed to continuous infection with *S. vulgaris* are incapable of mounting a protective immune response which may be accounted for by the immunosuppressive effects of polyclonal mitogenic activation (Bailey et al., 1984). A common feature of persistent parasitic conditions is often chronic hypergammaglobulinemia that may result from excessive mitogen-induced B

and/or T-cell proliferation. In vitro, T-cell cultures are stimulated to proliferate with *S. vulgaris* larval antigens while no proliferation is observed in B-cell enriched cultures. B-cells, however, do proliferate when both T-cells and *S. vulgaris* larval antigen are present. The stimulated cells have been shown to be a separate population from those stimulated by phytomitogens and LPS (Bailey et al., 1984). Characterization of the mitogen by gel filtration and ion-exchange chromatography showed that it was contained in the third and fourth fractions of the crude larval extract separated on Sephadex-G-200 and in the fourth fraction separated on DEAE-Sephadex A-25 (Adeyefa, 1992). In addition, the mitogenic fraction has been shown to be immunogenic by precipitation with IgG(T) from sera of horses naturally infected with *S. vulgaris* (Adeyefa, 1992).

Contrary to previous reports, recent studies have detected differences in lymphoproliferative responses between exposed and naive ponies. Significant differences in the blastogenic responses of both PBMC and CLNC to SAWA are observed when *S. vulgaris* challenged ponies are compared with parasite-free ponies (Swiderski, 1998a). Specific proliferative responses to SAWA have also been demonstrated in PBMC and mesenteric lymph nodes from ponies receiving trickle infections of 50 L3 per week for 25 weeks (Dennis et al., 1992). However, significant differences in blastogenesis between resistant and nonresistant ponies have not been detected before or after challenge (Klei, 1992). Recent observations, however, indicate a trend toward increased blastogenesis in IrrL3 vaccinates pre-challenge as well as 4 and 9 days post-challenge (Swiderski, 1998a).

Other specific cell-mediated immune responses to *S. vulgaris* antigens have been detected. For example, a cytokine or combination of cytokines that exhibits chemotactic activity for eosinophils was found in the supernatants of *S. vulgaris* stimulated PBMC and correlates with the anamnestic eosinophilia seen following *S. vulgaris* challenge (Dennis et al., 1993). This was the first proof that *S. vulgaris* infected ponies elicited an antigen specific cytokine response. Subsequent experiments suggested that this factor(s) is a heat sensitive glycoprotein, greater that 8000 kDa, and is produced specifically in response to *S. vulgaris* SAWA and not to *S. edentatus* antigens. These observations demonstrate a specific cellular immune response to *S. vulgaris* that is consistent with a Th2-like response.

IL-5 is a Th2 cytokine that is important for eosinophil growth, chemotaxis and activation. Correlating with the eosinophil-chemotactic factor and anamnestic eosinophilia reported in IrrL3 vaccinated ponies, these animals also demonstrate elevated IL-5 levels in PBMC 14 days following challenge (Swiderski et al., 1999a). IL-4, another Th2 cytokine, is also elevated in PBMC and CLNC of both IrrL3 vaccinated and naïve ponies following challenge with *S. vulgaris* (Swiderski et al., 1999b). Although both groups display increased IL-4 production, levels are higher in PBMC and CLNC of IrrL3 vaccinated ponies prior to challenge, and at days 4 and 9 in the CLNC and PBMC, respectively (Swiderski et al., 1999b). Nonvaccinated ponies did not demonstrate a significant increase in IL-4 production until day 9 in the CLNC. IL-4 is required for the generation and maintenance of antigen specific IgG and IgE production (Leonard, 1999). It is hypothesized that the elevated IL-4 levels in IrrL3 vaccinates prior to and 4 days following challenge may have elicited an early, local

IgG(T) or IgE response that helped mediate protection. IL-4 has also been shown to induce intestinal mastocytosis in rodent models (Madden et al., 1991), however, increases in mast cell numbers are not seen in the intestinal tissues of vaccinates or nonvaccinates 16 days post-challenge (Swiderski, 1999a).

In addition to the production of Th2 cytokines, protection in IrrL3 vaccinates also appears to be associated with the down regulation of Th1 cytokines (Swiderski et al., 1999b). Both IrrL3 vaccinates and nonvaccinates have decreased INF-γ production 9 days post-vaccination followed by a significant increase at day 16 (Swiderski et al., 1999b). Initially it was hypothesized that elevated IL-4 production in IrrL3 vaccinates may have accounted for the significant decrease in INF-γ on day 9. In the Th1/Th2 paradigm, inhibition of INF-γ production is often attributed to IL-10 (Leonard, 1999); however, recent studies in the horse have not shown a relationship between elevated IL-10 and reduced INF-γ (Swiderski et al., 1999b). Instead, both immune and naïve ponies demonstrate decreased IL-10 levels following challenge (Swiderski et al., 1999a). The Th1 cytokines INF-γ and IL-2 increased by day 14 in both vaccinates and nonvaccinates and possibly reflect inflammation and phagocytosis of dead parasites (Swiderski et al., 1999b). Further analysis reveals that the source of all these cytokines is a $CD4^+$ T-cell population (Horohov et al., unpublished observations). Necropsies performed 6 weeks following challenge with virulent *S. vulgaris* show significant periportal fibrosis in IrrL3 vaccinated ponies. It is currently hypothesized that IrrL3 vaccination induces the development of a Th2 $CD4^+$ T-cell response within the intestinal submucosa. This response leads to killing and phagocytosis of challenge larvae, clearance of dead larvae via the portal system and subsequent periportal

inflammation and fibrosis (Horohov et al., unpublished observations; Monahan et al., 1994).

In contrast to IrrL3 vaccination, immunization with Ribi-SAWA produces a strong but nonprotective antibody response to somatic adult and larval antigens (Monahan et al., 1994). Instead of protection, this immunization protocol exacerbates the arterial lesions and clinical signs resulting from challenge infection (Monahan et al., 1994). Contrasting with the IrrL3 vaccination, Ribi immunization does not elicit a Th2 cytokine profile but rather a marked production of INF-γ and reduced IL-4 and IL-5 in the CLNC and PBMC (Horohov et al, unpublished observations). Ribi-SAWA vaccinated ponies also fail to develop an eosinophilia or increased numbers of cecal eosinophils post-challenge. These difference in disparate immune responses between IrrL3 and Ribi-SAWA vaccinates is likely due to differences in Th1/Th2 cytokine production. These findings suggest that differential cytokine production to helminth infections may play an important role in conferring protective immunity to *S. vulgaris*.

CHAPTER 2[1]
ALTERED IMMUNE RESPONSES TO A HETEROLOGOUS PROTEIN IN PONIES WITH HEAVY GASTROINTESTINAL PARASITE BURDENS

Introduction

Helminth parasites have developed numerous mechanisms that enable them to avoid immune detection, recognition and clearance (reviewed by Riffkin *et al.* 1996 and Maizels *et al.* 1993). Nematode derived molecules may also represent a mechanism whereby parasites alter the normal regulation of the host immune response (Grencis and Entwistle, 1997) and consequently may alter the host response to additional antigenic stimuli. Several nematode species including *Ascaris suum*, *Nippostrongylus brasiliensis*, *Heligomosomoides polygyrus* and *Ancylostoma caninum* along with various filarial parasites have been shown to modulate the host immunity, as reviewed in Barriga (1984), Maizels (1993) and Riffkin *et al.* (1996).

Typically, gastrointestinal nematodes induce a Th2 cytokine response (reviewed in Else and Finkelman 1998). The polarization of the immune response towards Th2 cytokines by helminthes has been shown to down regulate or alter protective Th1 cytokine responses to bacterial and viral challenge. For example, the filarial parasites *Brugia malayi* and *Onchocerca volvulus* skew the classic Th1 immune response to *Mycobacterium tuberculosis* towards a mixed Th1/Th2 profile (Stewart *et al.* 1999; Pearlman *et al.* 1993). Mice coinfected with *Toxocara canis* and Japanese encephalitis virus have a diminished virus specific cellular and humoral immune response (Gupta and Pavri, 1987). Similarly, *Schistosoma mansoni* infected mice experience a decreased $CD8^+$ cytotoxic T cell response, reduced Th1 cytokine production and delayed vaccinia

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viral clearance (Actor *et al.* 1993). Limited equine studies have demonstrated that protection against the gastrointestinal nematode *Strongylus vulgaris* involves increased production of the Th2 cytokines IL-4 and IL-5 (Swiderski *et al.* 1999a; 1999b). The polarized Th2 character of the immune response in parasitised ponies may induce similar alterations in immunity to concurrent protein immunization.

Besides immunomodulation, helminth parasites can also exert multiple deleterious clinical and subclinical effects on a host that indirectly may alter the outcome of vaccination. For example, parasitism has been shown to cause inappetence (Coop and Holmes 1996; Mercer et al., 2000), alter feed utilization and metabolism, as well as decrease absorption of essential minerals (Mac Rae, 1993; Holmes, 1993). Coop and Kyriazakis (1999) argued that when nutrient resources are scarce, the expression of immunity to gastrointestinal parasites may yield to other bodily functions such as skeletal growth and lactation. It has been shown that protein losses occur during the course of parasitic infections (Coop and Holmes, 1996), and that decreased protein levels may compromise proteinaceous components of the immune system. Although these factors have not been studied directly in the horse, the consequences of internal parasitism are clinically apparent in regards to reduced weight gain and poor skeletal growth (reviewed in Slocombe, 1985).

Although horses under all management conditions are commonly infected with helminthes, there is currently no report on the effect of parasitism on subsequent antigenic challenge. In the current study, horses with high, medium and low levels of internal parasites were vaccinated with the protein keyhole limpet haemocyanin (KLH). Cytokine mRNA levels of IL-4, IL-5, INF-γ and IL-2 were measured using a

quantitative non-competitive RT-PCR procedure (Swiderski et al., 1998). Lymphoproliferative responses and serum antibody production to KLH antigen were also analyzed. The objective was to evaluate the effect that varying levels of gastrointestinal parasites have on the immune response to heterologous immunization in ponies. The hypothesis was that immunity to heterologous vaccination would be improved when the parasites were removed. Furthermore, it was expected that the response of parasitised ponies to KLH immunization would be polarized toward a Th2 cytokine profile.

Materials and Methods

Experimental Design

Twelve mixed breed, naturally infected ponies, ranging from one to three years of age were allocated to one of three treatment groups based on a stratified randomization of fecal strongyle egg counts. Although fecal egg counts may not reflect the ponies' mucosal larval burdens, this is the only technique available to estimate parasite load. The treatment groups were: 1. moxidectin 2% oral gel (Quest, Fort Dodge Animal Health, Fort Dodge, IA) (400 $\mu g\ kg^{-1}$) given once; 2. pyrantel pamoate (Strongid Paste, Pfizer Animal Health, New York, NY) (6.6 $mg\ kg^{-1}$) given twice at a three-week interval and 3. non-treated controls. It was expected that moxidectin would remove the intestinal nematodes, migrating large strongyles, stomach bots, and some mucosal stages of cyathostomes, while pyrantel pamoate would be partially effective and target only lumenal nematodes and tapeworms. To prevent parasite transmission during the experiment, ponies were housed in pairs on covered concrete paddocks and

fed a pelleted feed ration (Purina Horse Chow, Purina Feeds, St. Louis, MO) and hay. Water was provided *ad libitum*.

Twenty-four days following the first pyrantel pamoate treatment and single moxidectin treatment, all ponies were immunized with a single intramuscular injection of 2 mg KLH (Calbiochem Corp., San Diego, CA). Pre-vaccine, 2-week and 4-week serum samples were collected for antibody analysis and peripheral blood mononuclear cells (PBMCs) were collected at the pre-vaccine and 4-week time points for lymphoproliferation assays. The PBMCs were also collected at 4 weeks for cytokine analysis at which time all ponies were necropsied for parasite recovery and enumeration.

Necropsy Examinations

Ponies were humanely euthanized and complete necropsy examinations were performed as previously described (Klei and Torbert, 1980). The body cavity was opened and the intact viscera removed for parasite recovery. The cranial mesenteric artery (CMA) along with its major branches was dissected intact from the cecum and large intestine for enumeration of migrating *S. vulgaris* larvae. The cecum and large intestinal contents were collected separately including washings of the mucosal surface from each organ. A 1% aliquot of the total volume from the cecum and large intestine was fixed with 10% buffered formalin for parasite identification and enumeration. The remaining contents from the cecum and large intestine were sieved for recovery of large parasites. Stomach and small intestinal contents were also collected separately and sieved for recovery of large parasites. The total number of lumenal cyathostomes was determined by multiplying the number of parasites recovered from the 1% aliquot by a

factor of 100. HCl/pepsin digestion of the cecum and large colon was performed to determine the total number of mucosal cyathostomes (Monahan et al., 1996). The mucosal cyathostomes were identified as hypobiotic L3 (EL3) or developing L3 and L4 larvae (DL) (Chapman *et al.* 1999).

Preparation of Cells

Equine PBMCs were isolated from venous blood and prepared as described by Swiderski et al. (1998). The PBMCs were separated by differential centrifugation over Ficoll Paque (Amersham-Pharmacia, Piscataway, NJ) and washed three times in calcium and magnesium free phosphate buffered saline. After washing, the cells were suspended in RPMI 1640 (Sigma, St. Louis, MO) supplemented with HEPES, 2-mercaptoethanol (10^{-8} M), glutamine (2 mM), 100 U/ml penicillin, 100 μg/ml streptomycin and 5% heat inactivated fetal bovine serum (HyClone Laboratories, Logan, UT). Freshly isolated PBMCs were used immediately for lymphoproliferation assays. For cytokine quantification, PBMCs were incubated at 39°C in a humidified environment for 72 hours with 30 μg ml^{-1} KLH. Following incubation, aliquots of 3 x 10^6 PBMCs were frozen at –70°C in RNA Stat-60 (Tel-Test, Friendswood, TX) for later analysis.

Lymphoproliferation Assays

Equine PBMCs were cultured in triplicate at 2 x 10^5 cells per well with either 50 μg ml^{-1} KLH or 0.25 μg ml^{-1} pokeweed mitogen (PWM) and with or without the addition of 40 Units ml^{-1} of recombinant human IL-2. After a 5-day culture at 39°C in a 5% CO_2 humidified environment, cells were pulsed for 4 hours with 0.5 μCi of ^{3}H-thymidine and frozen at -20°C. Cells were harvested onto glass fiber filter pads and

counted by liquid scintillation (LKB Betaplate, Amersham-Pharmacia Biotech, Inc., Piscataway, NJ). Results are expressed as the stimulation index (SI), calculated as the mean count per minute (of triplicate wells) for antigen- or pokeweed mitogen-stimulated cells divided by the mean count per minute for unstimulated cells.

Cytokine Quantification

The PBMC mRNA levels of IL-4, IL-5, INF-γ, IL-2 and beta-actin were quantified by RT-PCR as described previously (Swiderski et al., 1998). Samples were thawed and the RNA was isolated using chloroform extraction. RNA levels were quantified by spectrophotometry and 1.2 μg of RNA was reverse-transcribed to complementary DNA (cDNA). The cDNA samples were amplified to 35 cycles along with standard curves that were generated by making half-log dilutions of plasmids containing the cytokine sequence of interest. Upstream primers were biotinylated on their 5' terminus and PCR reactions were hybridized to tris (2,2'-bipyridine) ruthenium II chelate labeled oligonucleotide probe whose sequence was specific to the cytokine targeted by PCR. Strepavidin-coated iron beads were added to each reaction and products were quantified on the QPCR System 5,000 (Perkin-Elmer, Foster City, CA) whose output is in luminosity units. The luminosity units for the PCR amplified plasmids were plotted against initial template numbers to yield a standard curve. Template numbers for specific cytokine cDNA in each sample were determined by interpolation from these standard curves and final values were reported as cytokine copy numbers. Upon completion each sample was normalized for RNA content using the values obtained for beta-actin. Results are expressed as the cytokine stimulation

index (CSI), calculated as the mean copy unit of cytokine for the antigen-stimulated cells divided by the mean copy unit of cytokine for the unstimulated cells.

Antibody Determination

ELISAs for serum antibody levels were performed as previously described (Swiderski et al., 1999a). Immulon I B (Dynex Tech., Chantilly, VA) flat bottom microtiter plates were coated overnight at 4°C with 20 μg/ml of KLH antigen. Coated plates were washed with PBS containing 0.05% Tween 20 in an automatic plate washer and blocked for 1 hour with PBS containing 1% fish gelatin (PBSG). Prevaccine, 2-week and 4-week post-vaccination serum samples were diluted 1:200 in blocking buffer and added to the wells in triplicate. After a 1 hour incubation at 39°C, monoclonal (Mab) anti-equine IgA, IgGa, IgGb or IgG(T) diluted 1:100 or horseradish peroxidase-conjugated anti-equine total IgG (Kirkegaard and Perry, Gaithersburg, MO) diluted 1:10,000 was added to the appropriate wells. After a second 1 hour incubation at 39°C, plates with Mabs were washed and incubated an additional hour with affinity-purified horseradish peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MO). Plates were developed with 3,3', 5,5'-tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MO) and analyzed at OD_{450}. ELISA units of each antibody isotype were determined by interpolation from a standard curve that was generated using serum from a known high responder.

Statistical Analysis

Data were analyzed by a one-way analysis of variance on ranks for the cytokine stimulation index, lymphoproliferation stimulation indices, and ELISA units. Differences were considered significant at $P < 0.05$ using the general linear model test

within the Statistical Analysis Systems (Statistical Analysis Systems Institute, Inc., Carey, NC) software.

Results

Necropsy Examinations and Parasite Recovery

The objective of this study was to investigate the effects of high, medium and low level internal parasitism on heterologous immunization in ponies. At necropsy, all lumenal parasites within the stomach, small intestine, and large intestine were enumerated. Encysted small strongyles were counted following HCl/pepsin digestion of tissue samples from the cecal, ventral colic and dorsal colic walls. Table 2.1 shows the mean number of parasite species recovered. In addition to these parasites, a single *Oxyuris equi* was recovered from a non-treated control pony and seven *Anoplocephala perfoliata* were recovered from a moxidectin treated animal.

Small strongyle species comprised the largest population of parasites recovered. The ponies in the moxidectin treated group (low parasites) had 99% fewer lumenal cyathostomes and 90% fewer mucosal cyathostomes than non-treated ponies. As expected, the pyrantel pamoate treated ponies (medium parasites) had a 75% and 79% reduction, respectively. Based on these results, the treatment protocol was successful in establishing ponies with a range of parasite numbers, which we designated as high (not treated), medium (pyrantel pamoate), and low (moxidectin) levels of differing internal parasites. Pyrantel pamoate was effective in treating only lumenal parasites whereas moxidectin was effective in treating lumenal parasites, most mucosal cyathostomes, and migrating *S. vulgaris*. This observation is consistent with previous broad-spectrum

Table 2.1. Total parasite recoveries from KLH-vaccinated ponies *.

Group	N	Cyathostome spp. ($x10^3$)				*Strongylus* spp.[a]	Arterial *S. vulgaris*[b]	*Parascaris equorum*	*Gastrophilus intestinalis*
		Mucosal		Lumenal					
		EL3	DL	L4	Adult				
Low Parasites	4	5.8 ± 4.8	1.7 ± 1.0	0.03 ± 0.05	0.3 ± 0.08	0	0	0	19.5 ± 15.6
Medium Parasites	4	16.2 ± 7.4	2.7 ± 1.8	1.7 ± 1.2	3.4 ± 2.7	1.5 ± 3[c]	19.3 ± 14.8	0.5 ± 0.6[d]	74.8 ± 70.1
High Parasites	4	72.0 ± 94.3	3.4 ± 1.7	2.0 ± 2.2	21.7 ± 7.5	82.3 ± 91.2	23.8 ± 9.9	13.8 ± 11.0	112.2 ± 74.6

*Mean ± standard deviation of 4 animals per treatment group.
[a] Includes lumenal adult *S. vulgaris* and *S. edentatus*.
[b] L4 and L5 stage *S. vulgaris* recovered from the CMA and associated branches.
[c] A single pony infected with 6 large strongyles.
[d] 2 ponies infected with 1 *P. equorum* each.

effects of moxidectin treatment against both lumenal and tissue dwelling parasites (Monahan et al., 1996).

Lymphoproliferative Response to KLH

To discern whether different levels of worm burden affect blastogenesis to a heterologous protein, PBMCs were isolated from venous blood prior to and four weeks following vaccination. The level of parasitism did not significantly affect the ability of PBMCs to proliferate in response to KLH stimulation (Figure 2.1). However, there was a trend toward an improved response in moxidectin treated ponies especially in comparison to pre-vaccine levels. PBMCs from all three treatment groups proliferated in response to the T and B cell mitogen PWM. The addition of 40 Units ml^{-1} recombinant human IL-2 did not affect blastogenesis (data not shown) indicating that IL-2 was not a rate-limiting factor in the assay.

Cytokine Production to KLH

The PBMC mRNA levels of IL-4, IL-5, INF-γ and IL-2 four weeks post-vaccination were compared within each treatment group (Figure 2.2). Values were also compared to non-KLH immunized, heavily parasitised ponies. Animals with the least parasites produced statistically significant higher levels of IL-4 compared with non-vaccinated, heavily parasitised ponies, and KLH-vaccinated, heavy and moderately parasitised ponies. Lightly parasitised ponies also demonstrated a trend towards increased INF-γ production compared to the other three treatment groups. No differences in IL-5 or IL-2 production were observed among the four treatment groups. Medium and heavily parasitised ponies produced similar cytokine levels as non-vaccinated animals.

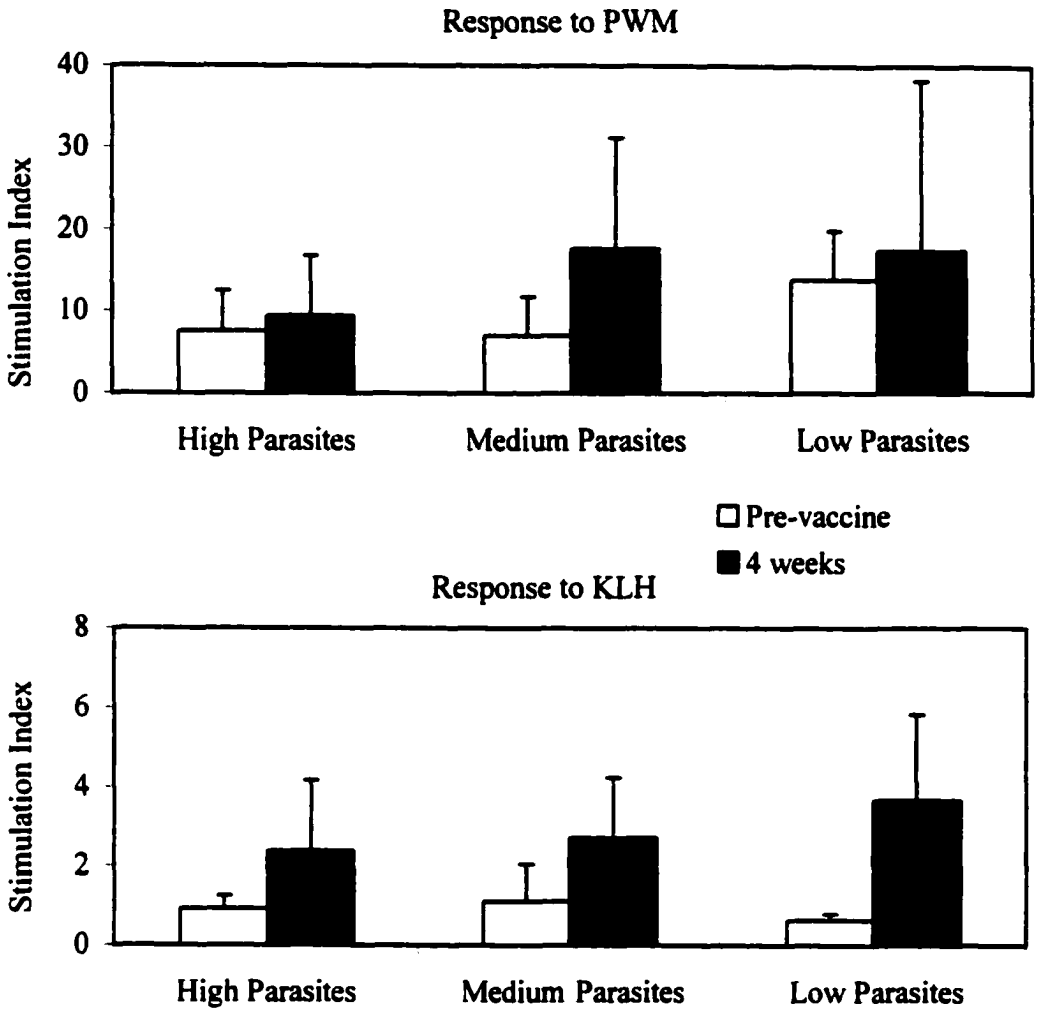

Figure 2.1. Lymphoproliferative response of pony peripheral blood mononuclear cells (PBMC) to mitogen and KLH stimulation. Following vaccination, PBMCs from ponies in all three treatment groups proliferate in response to KLH stimulation. Results are presented as the mean stimulation index and the error bars represent 1 standard error of the mean.

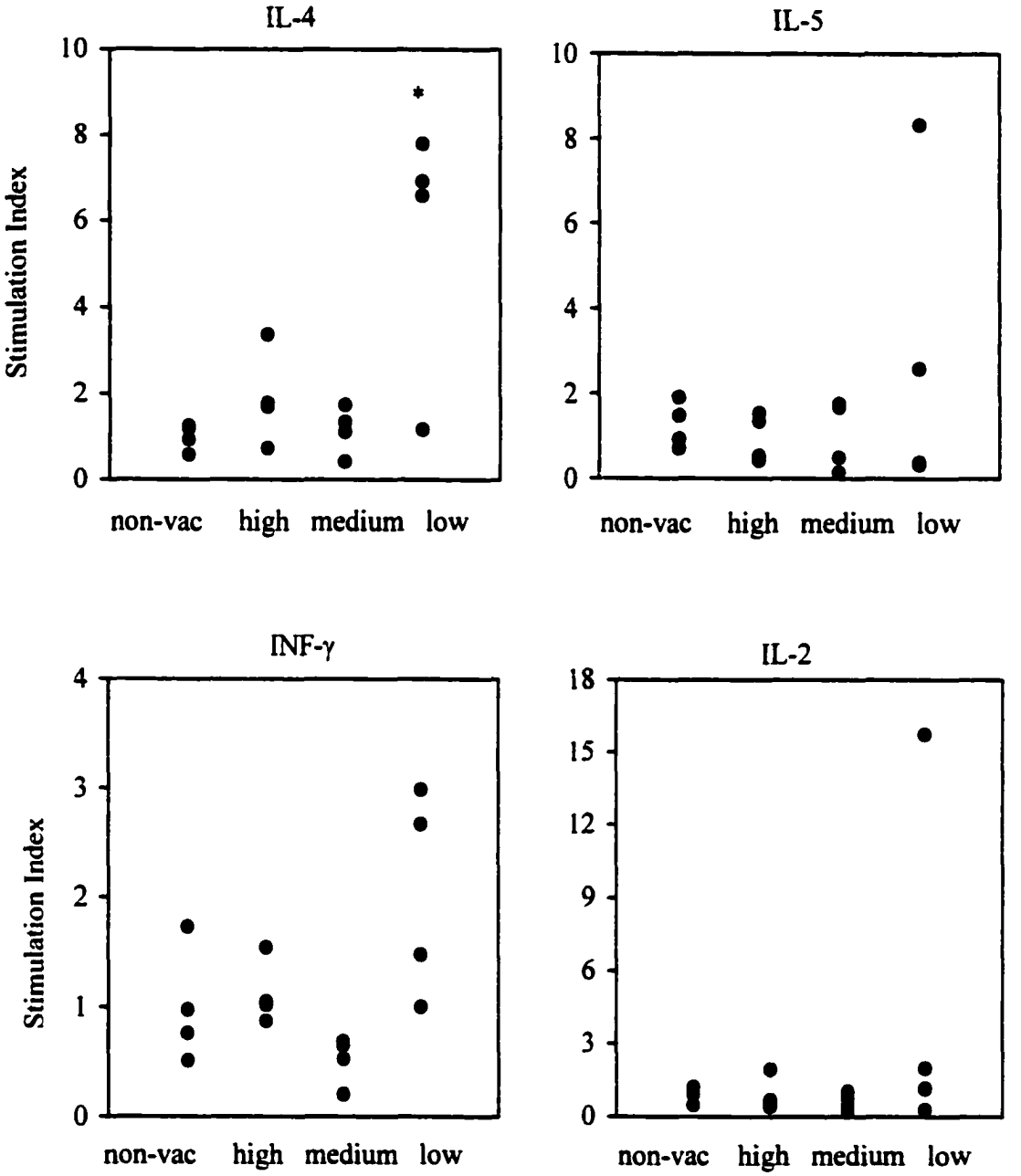

Figure 2.2. Cytokine stimulation index in non-KLH vaccinated ponies (non-vac) that were heavily parasitised or KLH vaccinated ponies with (high), (medium) or (low) gastrointestinal parasite burdens. Differences considered significant at $P < 0.05$, shown by *.

Antibody Production to KLH

While not statistically significant, ponies with low parasite burdens showed a trend towards increased levels of KLH specific total immunoglobulin and IgA two and four weeks following vaccination and a trend toward increased IgG(T) 2-weeks post-vaccination (Figure 2.3). Antibody levels were similar among the three treatment groups for the isotypes IgGa and IgGb (data not shown).

Discussion

In this report, the effect of internal parasitism on the cellular and humoral immune response to a non-parasite antigen, KLH, was analyzed. Protein-immunized ponies displayed a weak lymphoproliferative response to KLH stimulation regardless of the degree of parasite burden. KLH-induced proliferation was slightly higher in lightly parasitised ponies, particularly in comparison to pre-vaccine values, however the difference was not significant. Furthermore, PWM stimulation induced a similar level of proliferation in all three treatment groups. These data suggest that treatment for internal parasites may have a subtle effect on antigen but not mitogen induced lymphoproliferation. However, it is difficult in the current study to directly assess the role of parasites on the lymphoproliferative response. The experimental ponies were harboring natural infections consisting of different parasite genera, at different developmental stages and at different anatomical sites. Whereas previous studies report a parasite-induced immunosuppression to mitogen and antigen stimulation, these observations were made in animals experimentally challenged with one nematode species (Gasbarre, 1997; Crawford et al., 1989). Although more

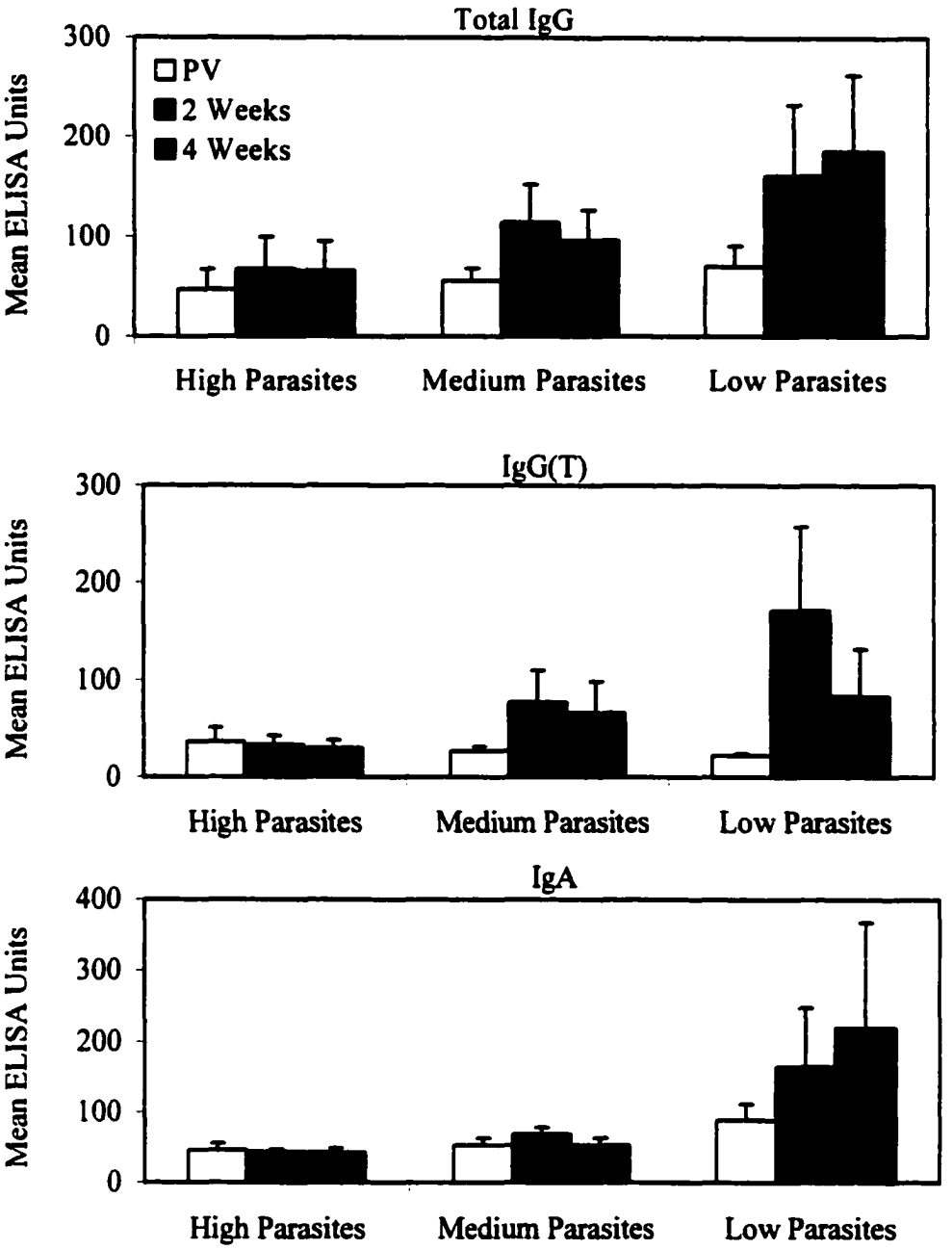

Figure 2.3. KLH specific total IgG, IgG(T) and IgA in ponies with high, medium or low gastrointestinal parasite burdens. Bars represent mean ELISA units for each treatment group of 4 at each collection time. Error bars represent 1 standard error of the mean.

variables are included with the current experimental design, the immune response is more reflective of a naturally parasitised animal. Also, the analysis of additional time points may have provided a more accurate indication of the presence or lack of blastogenic responses. In the current study, the lymphoproliferative response to KLH in all treatment groups was minimal. It is possible that although moxidectin eliminated >90% of the parasites, any level of helminth infection may reduce lymphocyte responsiveness. It is also possible that a greater KLH-specific lymphoproliferative response may have occurred with an increased KLH immunizing dose or with the addition of a booster vaccine.

During lymphoproliferation, clonal expansion of T cells is partially regulated by the availability of IL-2. In this study, cells were cultured with and without 40 Units/ml of recombinant human IL-2. The addition of IL-2 did not change the blastogenic response indicating that IL-2 was not a limiting factor in the assay. The retained mitogen stimulation suggests that the antigen hyporesponsiveness was not due to a lack of T-cell activation or T-cell numbers. However, these results do not account for a defect in antigen presentation or costimulation.

Previous work in ponies has shown that a Th2 cytokine profile characterized by the production of IL-4 and IL-5 is associated with protection against the intestinal nematode *S. vulgaris* (Swiderski et al., 1999a; 1999b). Conceivably, parasitised ponies exhibiting a dominant Th2 cytokine profile will also produce a biased Th2 response to heterologous immunization. This type of phenomenon has been reported in mice infected with the Th2 inducing *S. mansoni* and vaccinated with the heterologous protein, sperm whale myoglobin (SwMb). These mice have increased levels of SwMb

specific IL-4 and diminished INF-γ and IL-2 compared to uninfected mice (Kullburg *et al.* 1992). Also, the rat nematode *N. brasiliensis* induces a dominant Th2 immune response characterized by the production of IL-4, IgE and IgG1. When *N. brasiliensis* excretory/secretory proteins were co-administered with hen egg lysozyme (HEL) there was a HEL-specific lymphocyte proliferation, IL-4 release, and IgG1 antibody production. These studies illustrate the potential for helminth antigens to act as adjuvants for unrelated antigens (Holland *et al.* 2000). In the present study, medium and heavily parasitised ponies did not elicit a dominant Th1 or Th2 cytokine response to protein immunization. Instead, only the lightly parasitised ponies produced significantly higher IL-4 levels and displayed a trend towards increased INF-γ production. This increased cytokine production may reflect an overall improved immune responsiveness in lightly parasitised animals. Lack of a Th2 polarization may also have resulted from the low number of parasites present within the naturally infected ponies that had a migratory lifecycle. Since the majority of parasites present were cyathostomes, the local immune response within the cecum and large intestine may have been more Th2 biased than the systemic immune response.

Murine studies have also reported the effects of Th1/Th2 compartmentalization patterns. For example, mice infected with *S. mansoni* and immunized with SwMb, produce high levels of SwMb specific IL-4 in spleen cell cultures and none in lymph node cultures (Kullberg *et al.* 1992). In the current study, there may have been a greater Th2 bias in the colonic or mesenteric lymph node cells. Observations in our laboratory indicate that vaccinated ponies produce a more polarized Th2 response (IL-4 and IL-5)

in colonic lymph node cells compared to PBMCs 9 days following challenge with *S. vulgaris* (Horohov et al., unpublished observations).

In addition to the published effects of parasites on cellular immunity, parasitism has also been shown to alter humoral responses to heterologous vaccination. In this study, ponies with low parasite burdens demonstrated a trend toward increased levels of KLH-specific total immunoglobulin, IgG(T) and IgA. The observed increase in antibody production in the moxidectin treated animals also correlated with increased IL-4 secretion. While the biological function and cytokine regulation of equine immunoglobulin isotypes has not been characterized, an increase in serum IgG(T) has been reported following infections with *S. vulgaris* (Patton *et al.* 1978; Swiderski *et al.* 1999a) and *A. perfoliata* (Proudman and Trees 1996). IgA production has also been shown to increase following S. *vulgaris* challenge (Swiderski *et al.* 1999a). Conceivably, ponies relieved of their parasite burdens are still biased toward production of IgG(T) and IgA, and may elicit the same antibody profile to heterologous immunization. This trend towards increased antibody production in animals with the fewest parasites suggests an overall improvement in humoral immunity.

Immunosuppression and immunomodulation are two severe consequences that parasitism can impart on a host. In addition, helminth parasites can also cause other deleterious effects that may indirectly alter the outcome of heterologous vaccination. In general, GI nematodes reduce nutrient availability to the host through both reductions in voluntary feed intake and/or reductions in the efficiency of absorbed nutrients (Coop and Kyriazakis 1999). Clinically, this nutrient deficit is manifested by poor growth in young animals and loss of body weight in older animals. Gastrointestinal parasitism

increases protein and amino acid loss via the GI tract therefore reducing the amount of protein reserve available for other tissues (reviewed by MacRae 1993). The immune system must draw on these protein reserves for synthesis of most all of its components such as, immunoglobulins, cytokines, and products of mast cells, eosinophils, and neutrophils (MacRae 1993; Coop and Holmes 1996). Loss of essential amino acids and protein through the effects of gastrointestinal parasitism may compromise the host's ability to respond to additional antigenic stimuli and may explain the lack of immune responsiveness in the medium and heavily parasitised animals.

Clearly many factors are involved in measuring the immune response in parasitised animals to heterologous vaccination. Although several studies have been published on the effects of heterologous vaccination in rodent models (Stewart *et al.*, 1999; Pearlman *et al.*, 1993) this is the first report in the equine species. In conclusion, heterologous immunization of heavily parasitised ponies resulted in a trend towards a reduced cellular and humoral immune response. Although the presence of the parasites did not polarize the immune response towards a Th2 phenotype, overall immunity was improved when the parasites were removed. This effect may be demonstrated in horses that are managed for maximal parasite reduction and with the use of current antiparasiticides this can be accomplished (Klei 1997).

CHAPTER 3
VACCINE INDUCED ALTERATION OF THE EQUINE TH2 CYTOKINE RESPONSE TO *STRONGYLUS VULGARIS* RADIATION-ATTENUATED LARVAE

Introduction

Strongylus vulgaris is a highly pathogenic gastrointestinal nematode of equids, and has a well-defined lifecycle. Adults become established in the cecum and large intestine via extensive larval migration through the cranial mesenteric artery (reviewed by Ogbourne and Duncan, 1985). Larval migration within the intestinal vasculature can compromise perfusion of down-stream vascular beds through arterial damage or thromboembolism, a syndrome known as verminous arteritis or thromboembolic colic. This syndrome is characterized by ischemic infarction of the small and/or large intestine resulting in severe abdominal pain, endotoxemia and death (White et al., 1985). *S. vulgaris* was considered in the past to be the most important equine parasite due to a 70-90% prevalence rate and the severe consequences imparted by verminous arteritis (Ogbourne and Duncan, 1985). However, newer classes of anthelmintics (avermectin/ milbimycins) kill both migrating and adult stages of *S. vulgaris* and regular treatment with these drugs has dramatically reduced the rate of infection and disease (Klei, 1992). Despite this reduced incidence, *S. vulgaris* remains an important pathogen in areas that lack adequate parasite control. *S. vulgaris* is also a well-defined natural host-parasite system for experimental studies of equine immunity to helminths.

In this model system, experimental oral vaccination of parasite-free ponies with radiation-attenuated *S. vulgaris* L3 (IrrL3) significantly reduces larval burdens as well as prevents the classic lesions of verminous arteritis (Klei et al., 1982; 1986). Protection induced by IrrL3 vaccination is associated with the up-regulation of the T-

cell helper 2 (Th2) cytokines, interleukin-4 (IL-4) and IL-5 and the down-regulation of the Th1 cytokine INF-γ in both peripheral blood mononuclear cells (PBMC) and cecal lymph node cells (CLNC). However, differences between the Th1 cytokine, IL-2, and the Th2 cytokine, IL-10, were not detected before or after challenge infection (Swiderski et al., 1999a; 1999b). The Th2 pattern of cytokine production was confirmed in cells stimulated in vitro with *S. vulgaris* antigens and was shown to be mediated by $CD4^+$ T-cells (Horohov et al., unpublished observations). Correlating with increased levels of IL-5, IrrL3 vaccinated ponies elicit an anamnestic eosinophilia 1 to 2 weeks following virulent challenge (Swiderski et al., 1998a; Monahan et al., 1994). Compared to nonimmune animals there is also a significant increase in intestinal eosinophils on day 16 (D 16) (Horohov et al., unpublished information) and a significant increase in intestinal and cranial mesenteric eosinophils on D 35 (Monahan et al., 1994).

In contrast to IrrL3 vaccination, intra-muscular immunization with soluble somatic extracts of adult *S. vulgaris* in combination with Ribi adjuvant (Ribi-SAWA) is not protective. Ponies administered Ribi-SAWA exhibit an exacerbation of arterial lesions and clinical signs following challenge (Monahan et al., 1994). Instead of inducing Th2 cytokines as seen with IrrL3 vaccination, Ribi-SAWA immunization induces a marked production of INF-γ and reduced IL-4 and IL-5 in the CLNC and PBMC (Horohov et al, unpublished observations). Ribi-SAWA vaccinates also fail to develop an eosinophilia or increased numbers of cecal eosinophils post-challenge. These differences in immune responses between IrrL3 and Ribi-SAWA vaccinates are likely due to the disparate Th1/Th2 cytokines produced following vaccination with the

two different immunization regimes. Further, these findings suggest that Th2 cytokines play an important role in conferring protective immunity to *S. vulgaris* in the horse.

The objective of the current study was to investigate in equids the potential cross regulation of Th1 cytokines on the induction of Th2 cytokines and to demonstrate the importance of the Th2 response for protective immunity to *S. vulgaris*. To accomplish these objectives, ponies were sensitized with the Th1-inducing Ribi-SAWA prior to and concurrently with the Th2-inducing IrrL3 immunization. Ponies were challenged and then monitored for 28 days to determine the effect of the Th1 cytokines potentially produced by the Ribi vaccination on the pathology and clinical signs associated with *S. vulgaris*. The hypothesis was that in a milieu of Th1 cytokines, vaccination with IrrL3 would not induce a dominant Th2 cytokine response and ponies would be susceptible to *S. vulgaris* challenge.

Materials and Methods

Experimental Design

Seventeen yearling ponies raised and maintained in parasite-free conditions (Monahan et al., 1994) were used. Pairs of ponies were housed in concrete stalls on wood shavings, fed a pelleted feed ration (Purina Horse Chow, Purina Feeds, St. Louis, MO), and were provided water *ad libitum*. Ponies were allocated to one of five treatment groups (Table 3.1). Ponies in the first treatment group were vaccinated intramuscularly with Ribi-SAWA three weeks (D -84) prior to the concurrent immunization (D -63) with Ribi-SAWA and orally administered IrrL3. A second IrrL3 vaccine and third Ribi-SAWA vaccine was administered after an additional three weeks

Table 3.1. Treatment group allocation of nematode-naïve pony foals.

Treatment Group	N	Day -84	Day -63	Day -42	Day 0	Day 28
R[1]+I[2] +C[3]	3	Ribi-SAWA	Ribi-SAWA & IrrL3	Ribi-SAWA & IrrL3	Challenge	Necropsy
I+C	4	-	IrrL3	IrrL3	Challenge	Necropsy
R+C	4	Ribi-SAWA	Ribi-SAWA	Ribi-SAWA	Challenge	Necropsy
N[4]+C	4	-	-	-	Challenge	Necropsy
N	2	-	-	-	-	Necropsy

[1]R – Ribi-SAWA, 1mg soluble adult *S. vulgaris* protein with Ribi adjuvant inoculated intramuscularly.
[2]I – IrrL3, 500 third stage larvae of *S. vulgaris* exposed to 90kRads of Co^{60} given orally.
[3]C – Challenge, 1000 *S. vulgaris* given orally.
[4]N – Naïve.

(D -42). On D 0, six-weeks following the last immunization, ponies were orally challenged with 1000 non-irradiated *S. vulgaris* L3. These ponies are designated as R+I+C. The remaining four treatment groups served as controls and were as follows: (I+C) IrrL3 given twice at a three-week interval and challenged; (R+C) Ribi + SAWA given three times at a three-week interval and challenged; (N+C) nonvaccinated and challenged; (N) nonvaccinated and nonchallenged ponies. Only three ponies were included in the R+I+C group since one pony died prior to D 0 from causes unrelated to the vaccination protocol. On D -5 and D 9 cecal lymph node cells (CLNC) were collected via a ventral midline ceileotomy for cytokine quantification as previously described (McClure et al., 1994; Swiderski et al., 1999b). All ponies were humanely euthanized on D 28 at which time CLNC were collected again, tissue samples were taken for histology, and challenge larvae were recovered. Blood was also drawn on D -5, D 9 and D 28 for the collection of peripheral blood mononuclear cells (PBMC) for further cytokine analysis.

Measurement of Clinical Signs

Ponies were monitored daily during the vaccination and challenge period for signs of depression, anorexia and colic. Rectal temperatures were measured for 2 days following the first and second IrrL3 vaccination and then daily starting 4 days prior to challenge and continuing daily throughout the challenge period. Ponies were considered pyrexic when rectal temperature exceeded 39°C. During daily evaluations the ponies' attitudes were assessed. Ponies were considered depressed if they showed the following signs: standing with a lowered head and drooped ears, displaying a dull facial expression and responding slowly to direct stimulation. Colic was diagnosed

when ponies had visible signs of abdominal pain such as pawing/kicking at the abdomen, looking back at the flank, repeatedly getting up and down, and/or rolling from side to side. Body weights were recorded on D -5 and D 28 and results are expressed as the mean weight loss and percent weight loss. The percent weight loss was calculated by dividing the mean D 28 weight by the mean D -5 weight and multiplying by 100.

Parasites and Parasite Antigens

The *S. vulgaris* L3 used for both vaccination and challenge were obtained from Baermann sedimentation of fecal cultures from monospecifically infected donor ponies (McClure et al., 1994). Following harvest from the Baermann apparatus, larvae were washed in tap water and stored at 4°C. Each IrrL3 vaccination consisted of 500 *S. vulgaris* L3 irradiated with 90 kRads Co^{60} according to a previously established protocol (Klei et al., 1982). Each vaccine was administered orally by syringe and given twice over a three-week interval.

The SAWA used for immunizations was prepared from adult male and female *S. vulgaris* collected from the cecum and large intestine of infected ponies as previously described (Monohan et al., 1994). Each immunization consisted of 1mg/ml of SAWA emulsified with Ribi adjuvant (MPL+TDM+CWS Emulsion R-730, Ribi ImmunoChem Research, Inc. Hamilton, MT) as recommended by the manufacturer. Intramuscular immunizations were given at 3-week intervals and administration of the last 2 Ribi-SAWA vaccines were given concurrently with the two IrrL3 vaccines.

Hematology

Blood samples were collected weekly following challenge for CBC analysis. Differential white cell counts were determined at 100x by counting 100 cells.

Necropsy Examinations and Larval Recovery.

Complete necropsy examinations were performed as previously described (Klei and Torbert, 1980). Briefly, the body cavity was opened and the intact viscera removed. Lesions were recorded and representative samples were taken from the ileo-ceco-colic artery (ICC), cecum, and liver for histologic analysis. The cranial mesenteric artery (CMA) along with its major branches was dissected intact from the cecum and large intestine for examination and enumeration of migrating *S. vulgaris* larvae (Klei et al., 1982). The lumen of each vessel was scraped to remove the intima, which was then examined under a dissecting microscope for recovery of challenge larvae.

Arterial Dissection

The CMA and associated branches were opened and evaluated for changes in the aorta, CMA, ICC, ventral colic, dorsal colic and cecal arteries. The ICC arteries were examined grossly and a score was assigned for thickening, intimal tracts, thrombosis, and aneurysm development as previously described (Klei et al., 1986). Intimal tracts were scored as follows: 0- no tracts; 1- few distinct tracts; 2- several tracts that coalesce in a few focal areas; 3- many tracts that coalesce in several areas; and 4 coalesced tracts covering the entire lumenal surface of the artery. Thrombus formation was scored as follows: 0- no thrombus; 1- small, focal thrombi; 2- small, multifocal thrombi; 3- large, multifocal thrombi; and 4- large thrombi occupying the majority of the arterial lumen. Thickening of the arterial wall was scored 0 for no thickening; 1- for mild focal thickening; 2- diffuse mild thickening; 3- diffuse, fibrous thickening with loss of vessel pliability; and 4- if the vessel wall was rigid and calcified. Aneurysm formation was scored as "0" for no aneurysm; "early" if there were small areas of

lumen dilation with pocket formation; "intermediate" if there were numerous pockets and overall lumen dilation due to diverticuli; and "late' if these diverticuli had coalesced into a large cavernous aneurysm.

Histology

Tissue samples from the liver, cecum, and ICC were fixed in buffered 10% formalin and embedded in paraffin. Each sample was stained with hematoxylin and eosin, Giemsa for eosinophil quantification, and toluidine blue for mast cell enumeration. Giemsa and toluidine blue stained cecal tissue sections were examined under high power with conventional light microscopy to identify regions of inflammation within the submucosa and lamina propria. Cecal eosinophils and mast cells were counted in five inflamed fields (40x) and five noninflamed fields (40x) within the submucosa and lamina propria. Results are expressed as the mean number of eosinophils/mast cells in both inflamed and noninflamed high power fields.

Hepatic tissue samples were analyzed on hematoxylin and eosin stained slides and lesions were scored as follows: 0- normal, 1- mild periportal inflammation with a small infiltration of eosinophils and mild lymphocytic cuffing (Figure 3.1A), 2- clearly delimited areas of moderate periportal inflammation and fibrosis with a marked eosinophil and mononuclear cell infiltration. (Figure 3.1B), 3- severe and expansive periportal eosinophilic inflammation that invaded adjacent parenchyma (Figure 3.1C). Lesions that scored a 3 showed pronounced biliary hyperplasia, fibrosis and local necrosis.

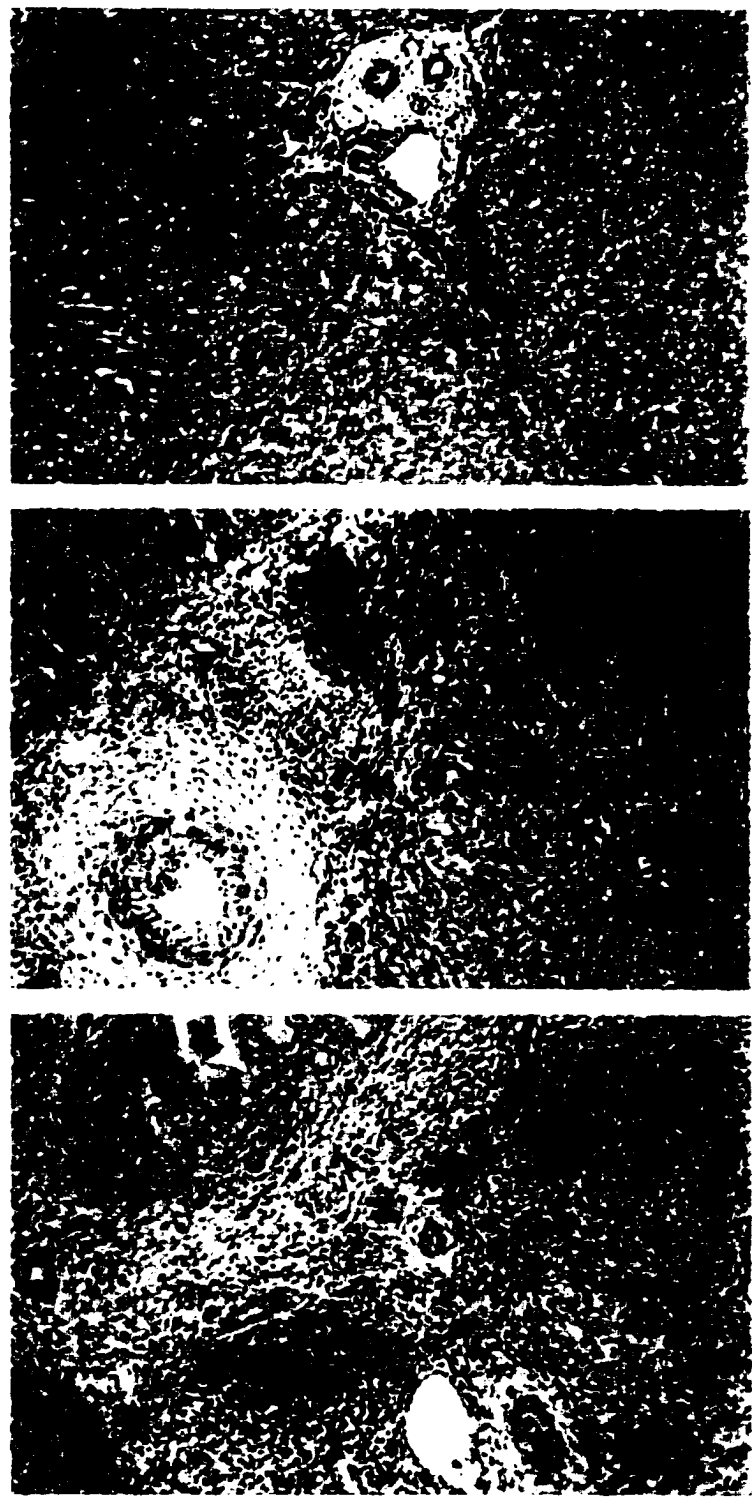

Figure 3.1. Hepatic lesions induced by migration of *S. vulgaris* in pony liver. (A) Score 1, mild lymphocytic and eosinophilic periportal cuffing; (B) Score 2, delimited areas of moderate periportal fibrosis with a marked eosinophil and mononuclear cell infiltrate; (C) Score 3, severe and expansive periportal fibrosis and necrosis with marked lymphoid development and infiltration of degranulated eosinophils.

Preparation of Cells

Equine PBMC were isolated from venous blood and prepared using the technique described by Swiderski et al. (1999a). Briefly, the PBMC were separated by density gradient centrifugation over Ficoll Paque (Amersham-Pharmacia, Piscataway, NJ) and washed three times in calcium and magnesium free phosphate buffered saline (CMF-PBS). A single cell suspension of equine CLNC was made by disruption through a sterile wire mesh screen followed by three washes in CMF-PBS. Aliquots of 3×10^6 PBMC and CLNC were frozen at –70°C in RNA Stat-60 (Tel-Test, Friendswood, TX) for cytokine quantification assays.

Cytokine Quantification

The PBMC and CLNC mRNA levels of IL-4, IL-5, IL-13, INF-γ, and β-actin were quantified as described in Chapter 2.

Statistical Analysis

Statistical analysis was performed using Sigma Stat software (Chicago, IL). The number of days that ponies demonstrated anorexia, pyrexia, depression and colic, along with weight loss, larvae recovery, ICC lesion scores, and hepatic lesion scores were analyzed by a one way analysis of variance (ANOVA). Peripheral eosinophil counts, submucosal eosinophils, lamina propria eosinophils and submucosal mast cells were also subjected to a one way ANOVA. When values were significant, all pairwise comparisons were made using a Student-Newman-Keuls test. Cytokine copy numbers were log transformed in order to normalize the data. Log transformed cytokine copy units were then analyzed by a repeated measures one way ANOVA and also analyzed at each time-point by a one-way ANOVA. When differences were significant between

time-points, all pairwise comparisons were made using the Student-Newman-Keuls test. Differences were considered significant at $P < 0.05$.

Results

Clinical Response

During the pre-challenge period, the R+I+C ponies had a more severe clinical response to vaccination compared to ponies in the other treatment groups. Two out of three ponies in the R+I+C group were febrile 2 days following the first IrrL3 vaccination and one pony was depressed five days following this first IrrL3 administration. In addition, one R+I+C pony was febrile 2 days following the second IrrL3 vaccination. Only 2 of 4 ponies in the R+C group were febrile 1 day following the third Ribi-SAWA vaccine and one of these ponies also exhibited mild signs of colic. Ponies in the I+C, N+C and N groups did not exhibit pyrexia prior to challenge (Figure 3.2.). One pony in the I+C group exhibited mild colic for 2 days following the second IrrL3 vaccine while ponies in the N+C and N groups did not demonstrate any abnormal clinical behavior during the pre-challenge period (data not shown).

Following challenge, ponies in the N+C group were depressed significantly more days than ponies in the other four treatment groups. These ponies also showed significantly more days of pyrexia than ponies in the R+I+C group (Table 3.2.). All challenged animals were febrile two days following infection with *S. vulgaris*. This febrile response correlated with larval penetration of the intestinal mucosa as previously reported (Monahan et al., 1994). The N+C and R+C ponies also demonstrated a second febrile response that was sustained throughout the challenge period and correlated with larval arterial migration (Monahan et al., 1994). The observed pyrexia of the I+C and

Figure 3.2. Mean pony rectal temperature in °C pre- and post-challenge. Temperatures were measured 2 days after the first (Day -63) and second radiation-attenuated L3 (IrrL3) immunizations (Day -42), 4 days prior to challenge and daily following challenge (Day 0) with 1000 *S. vulgaris*.

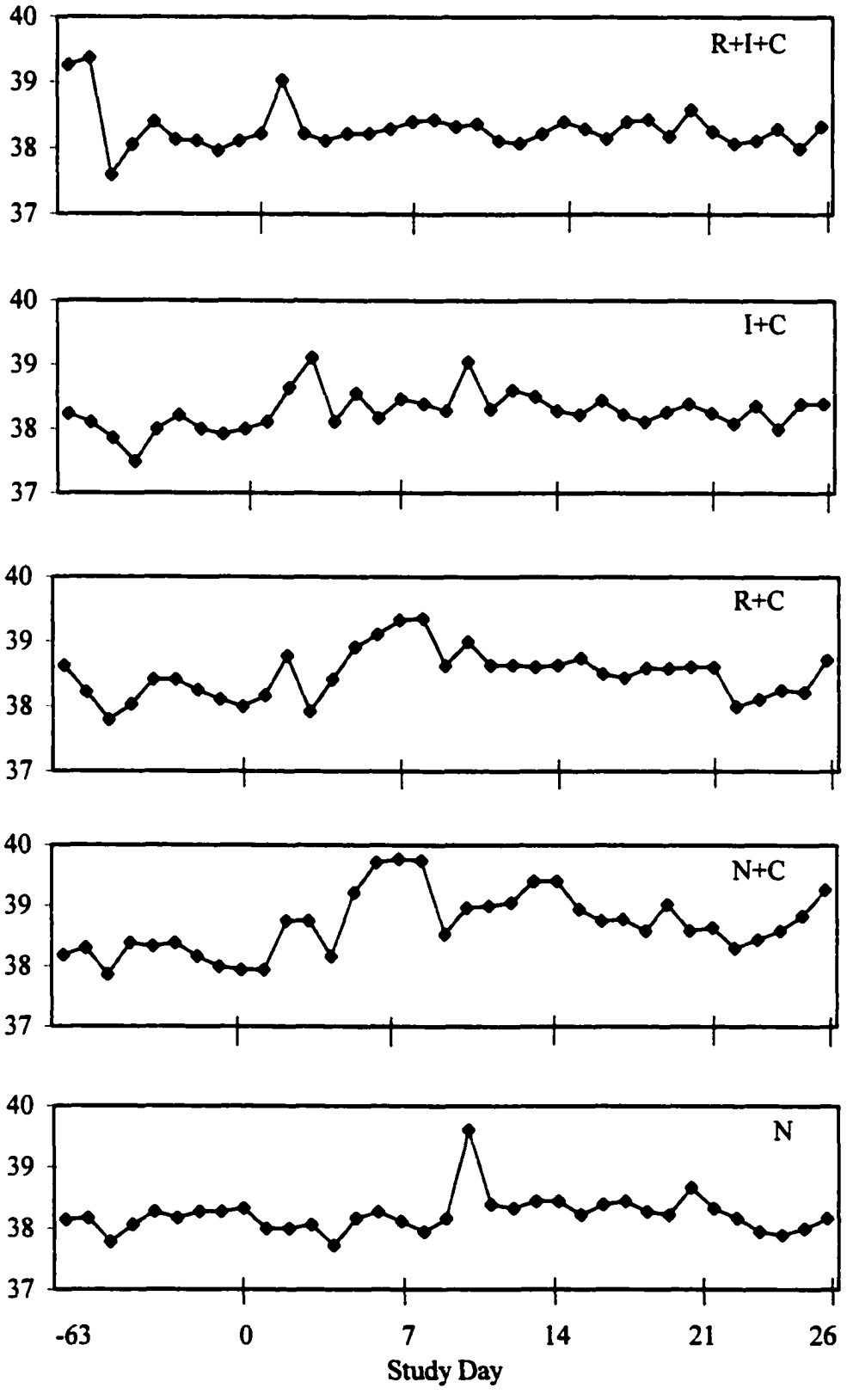

Table 3.2. Clinical signs exhibited in ponies following challenge with 1000 *S. vulgaris* L3.

Treatment Group	N	Anorexia	Pyrexia	Depression	Colic
R+I +C	3	1.7*[a] (0-3)	1.0[b] (0-2)	0.7[b] (0-2)	0[a]
I+C	4	4.3[a] (0-7)	3.8[a,b] (1-6)	0.3[b] (0-1)	0[a]
R+C	4	7[a] (0-14)	4.5[a,b] (1-9)	0.5[b] (0-1)	0.3[a] (0-1)
N+C	4	11.5[a] (7-16)	8.3[a] (6-10)	3.0[a] (2-5)	0.8[a] (0-3)
N	2	2.0[a]	0.5[b] (1-2)	1.0[b]	0[a]

*Mean number of days ponies showed this response.
( ) The range of days that ponies exhibited the respective clinical sign.
([a,b]) Values with the different superscript are statistically different.

N ponies on D 10 was likely related to the surgery performed on D 9. There was no difference in the number of days that anorexia and colic was exhibited, however, R+C and N+C ponies demonstrated a trend towards a greater number of anorexic and colic days compared to R+I+C, I+C, and N ponies. The total weight loss and percent weight loss that occurred following challenge are summarized in Table 3.3. The N+C and R+C ponies lost significantly more weight compared to ponies in the R+I+C and I+C groups.

Hematology

Differential WBC counts on weekly blood smears revealed that the I+C ponies developed an anamnestic eosinophilia on D 9 and elevated levels were sustained through D 28 (Figure 3.3). The R+I+C ponies also exhibited an anamnestic-like eosinophil response, however, there was a trend in this response towards reduced and delayed eosinophil numbers compared to I+C ponies. Eosinophils in the N+C group were elevated on D 21 whereas R+C eosinophils were not elevated until D 28. Naïve ponies demonstrated no eosinophilia at any time during the challenge period.

Larval Recovery

Ponies in the R+I+C and I+C groups demonstrated a significant reduction in migrating larvae compared to R+C and N+C ponies (Table 3.4.). There was not a significant difference in larval numbers between N+C and R+C animals although R+C did demonstrate a 25.3% reduction in migrating larvae compared to N+C animals.

Arterial Dissection

The R+C and N+C ponies displayed lesions typical of verminous arteritis (Duncan and Pirie, 1972; McCraw and Slocombe, 1976). The majority of the arterial lesions were present within the ICC artery and average ICC lesion scores are

Table 3.3. Mean and percent weight loss in ponies following *S. vulgaris* challenge.

Treatment Group	N	Weight Loss (kg)	Percent Weight Loss
R+I+C	3	3.0[a]	1.44
I+C	4	3.6[a]	3.33
R+C	4	7.5[b]	6.34
N+C	4	11.5[b]	10.03
N	2	1.0[a]	0.83

([a,b]) Values with the different superscript are statistically different.

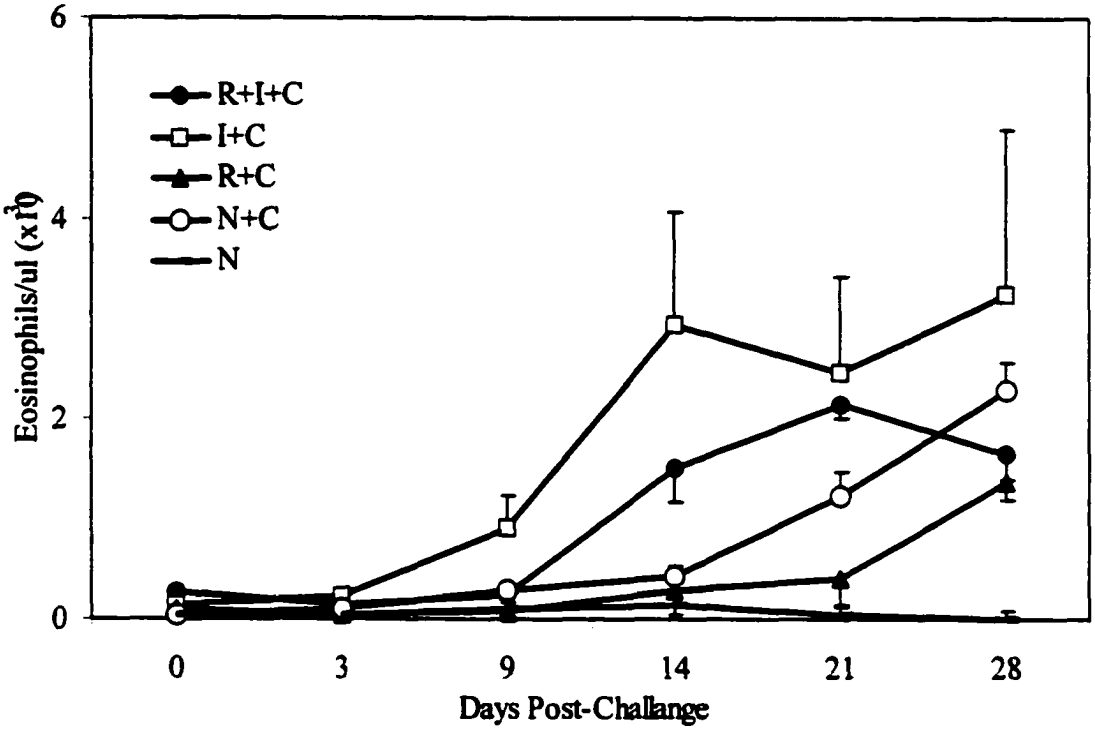

Figure 3.3. Eosinophil response in ponies to *S. vulgaris* challenge. Error bars represent ± 1 standard deviation.

Table 3.4. *S. vulgaris* L4 recovered from pony mesenteric artery dissections.

Treatment Group	N	Larvae Recovered[1]	Percent Protection[2]
R+I+C	3	8.7 ± 7.0^{a} (2-16)	93.1
I+C	4	10.8 ± 13.7^{a} (1-30)	88.6
R+C	4	70.3 ± 41.6^{b} (12-103)	25.3
N+C	4	94.0 ± 31.2^{b} (55-131)	-
N	2	0^{b}	-

Larvae were recovered from scrapings of the lumen of the CMA and its branches
* Mean ± standard deviation.
( ) Range of larvae recovered from individual animals.
Percent protection was calculated by the formula [(1-(total larvae recovered from arteries of immunization group/total larvae recovered from arteries of Naïve + Ch)) x 100].
(^{a,b}) Values with the different superscript are statistically different.

summarized in Table 3.5. Arterial changes included moderate to severe thickening of the vessel wall, mild to moderate thrombus formation and early aneurysm development. Intimal tracts were typically not visible in the arteries of the R+C and N+C ponies due to severe intimal erosion. The mesenteric arteries of the R+I+C had very few arterial changes and were similar to parasite-free ponies with the exception of mild thickening and a few intimal larval tracts. The I+C ponies also displayed few arterial lesions and while not statistically significant, these animals appeared to have more larval tracts than the R+I+C animals.

Histology

The R+C and N ponies had significantly fewer cecal eosinophils in the submucosa compared to R+I+C, I+C and N+C ponies and significantly fewer eosinophils in the lamina propria than ponies in the R+I+C and I+C groups (Figure 3.4). The numbers of submucosal and lamina propria eosinophils in R+C ponies were not significantly different from naïve animals. In all five treatment groups, mast cells were not visualized in the lamina propria of ponies and only low numbers were counted in the submucosa. There was not a significant difference in mast cell numbers found in the five treatment groups (Figure 3.5).

The ICC artery was chosen for histologic analysis since this is usually the most severely effected arterial site in *S. vulgaris* infected equids (Duncan and Pirie, 1972). Ponies in the R+C and N+C groups had lesions consistent with verminous arteritis. These lesions have been previously described (Duncan and Pirie, 1972; McCraw and Slocombe, 1976) and included severe destruction of the normal arterial architecture,

Table 3.5. Average pony ileo-cecal-colic artery and hepatic lesion scores.

Treatment Group	N	Thickness	Larval Tracts	Thrombus Formation	Aneurysms	Hepatic Lesion
R+I+C	3	1.5* (1-2)	1.0 (1)	0	0	3.0 (3)
I+C	4	1.0 (1)	1.3 (0-4)	0.5 (0-2)	0	2.0 (1-3)
R+C	4	2.1 (1-3.5)	0.6 (0-2.5)	2.4 (0-3)	Early	0.5 (0-1)
N+C	4	1.8 (0-3)	0	2.9 (2.5-3)	Early	0.3 (0-1)
N	2	0	0	0	0	0 (0)

* Data represents the mean ICC artery and hepatic lesion score that was assigned following gross dissection on D 28.
( ) Range of scores assigned to individuals within the respective treatment group.

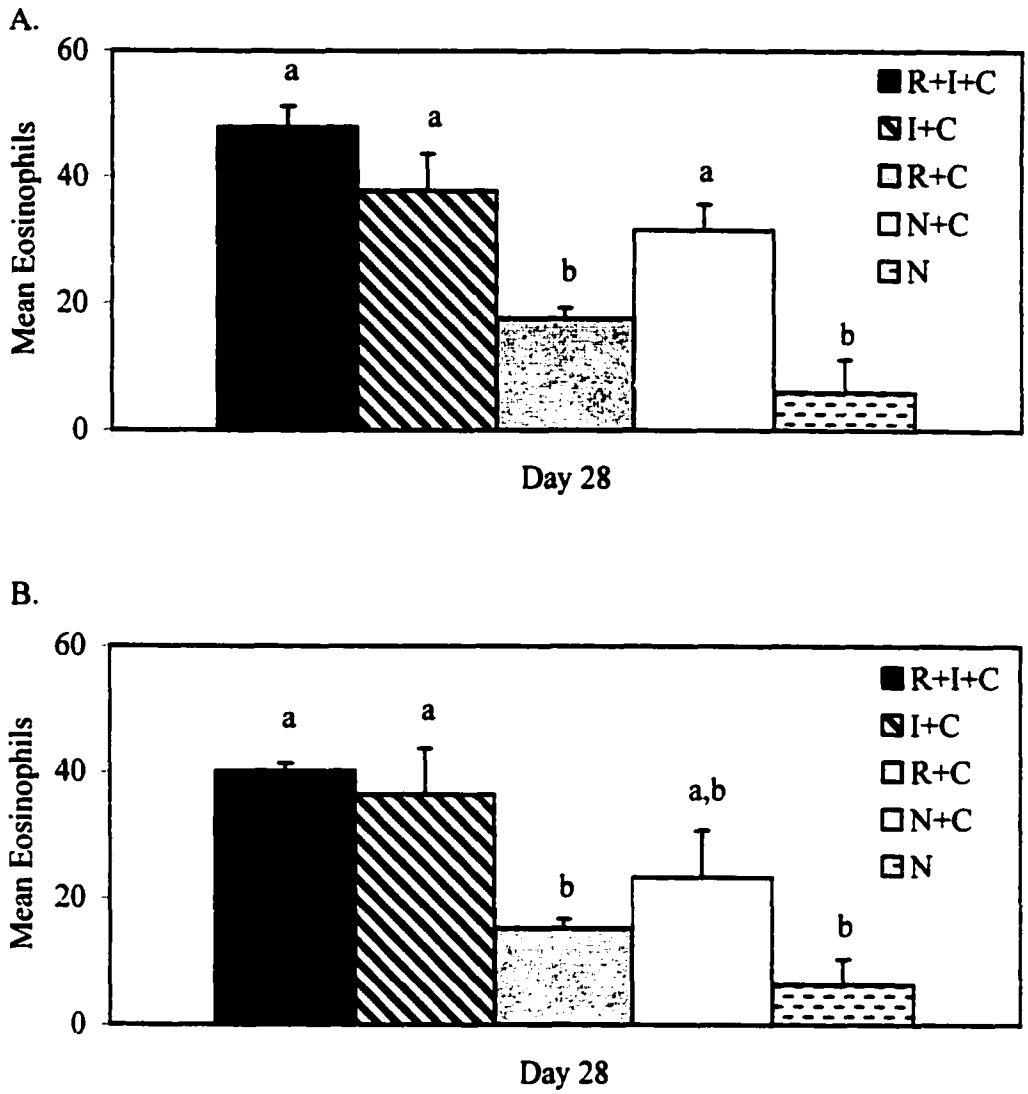

Figure 3.4. Mean eosinophil counts per high power field in the A. submucosa and B. lamina propria on day 28 following *S. vulgaris* challenge. Error bars represent 1 standard error of the mean. (a,b) Values with the different superscript are statistically different.

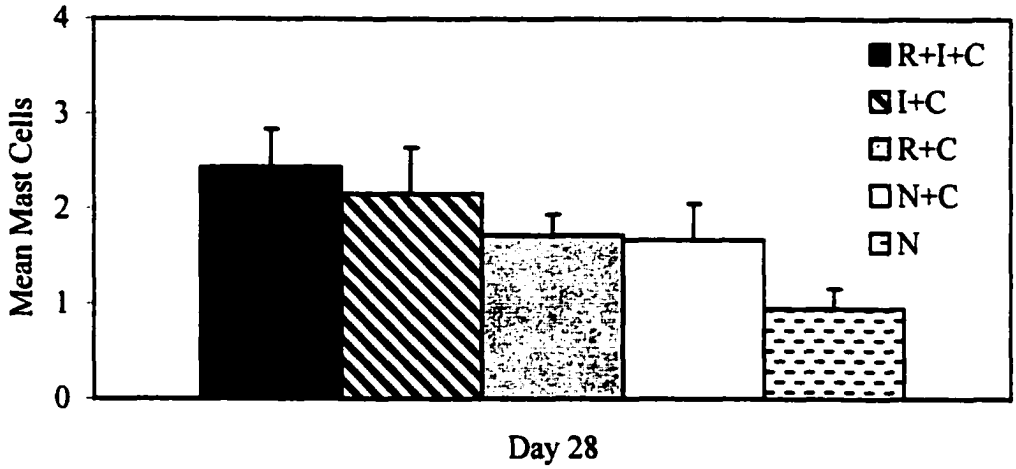

Figure 3.5. Mean mast cell counts per high power field in the submucosa on day 28 following *S. vulgaris* challenge. Error bars represent 1 standard error of the mean.

thrombus formation, loss of the intimal lining, fibrosis and neovascularization of the arterial intima, transmural lymphoplasmocytic infiltration, and eosinophilic infiltration of the arterial adventitia. In contrast, ponies in the R+I+C and I+C groups retained normal arterial architecture and the intimal lining remained intact. The R+I+C and I+C ponies also demonstrated a trend toward increased eosinophil infiltration and nodular lymphoid development within the arterial adventitia. These findings are consistent with the arterial lesions described in previously published studies of IrrL3 vaccinated ponies (Monahan et al., 1994).

Average lesion scores of hepatic tissue sections are summarized in Table 3.5. Ponies in the R+I+C group had a higher average score than the other four treatment groups. Although periportal fibrosis was also exhibited in the I+C ponies, the response was not as severe as seen in R+I+C ponies. However, the eosinophils within the periportal regions of both R+I+C and I+C ponies showed evidence of degranulation. The degranulated eosinophils had disrupted cytoplasms and their granules had spilled out into the tissue. Two ponies in the R+C group and one N+C animal demonstrated a mild eosinophilic periportal inflammatory response, although there was no evidence of eosinophil degranulation.

Cytokine Quantification

Cytokine copy units of IL-4, IL-5, IL-13 and INF-γ in CLNC (Figure 3.6) and PBMC (Figure 3.7) were quantified on D -5, D 9, and D 28. All challenged ponies had significantly elevated levels of IL-4 mRNA on D 9 and D 28 in CLNC compared to pre-challenge levels. The I+C and to a lesser degree the R+I+C ponies demonstrated a

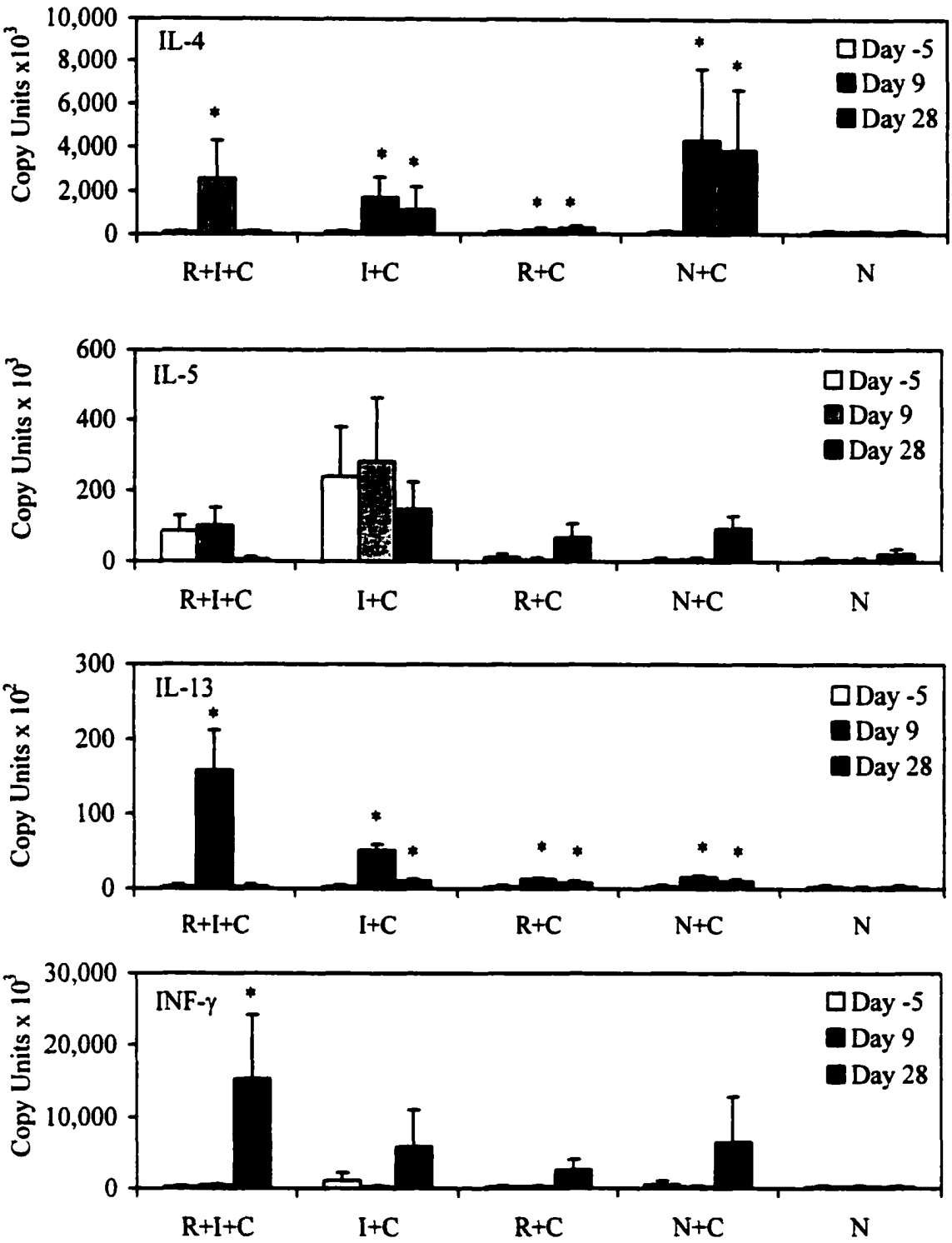

Figure 3.6. Cytokine copy units in pony cecal lymph node cells (CLNC). * Cytokine levels are significantly greater than pre-challenge (Day -5) levels. Error bars represent 1 standard error of the mean.

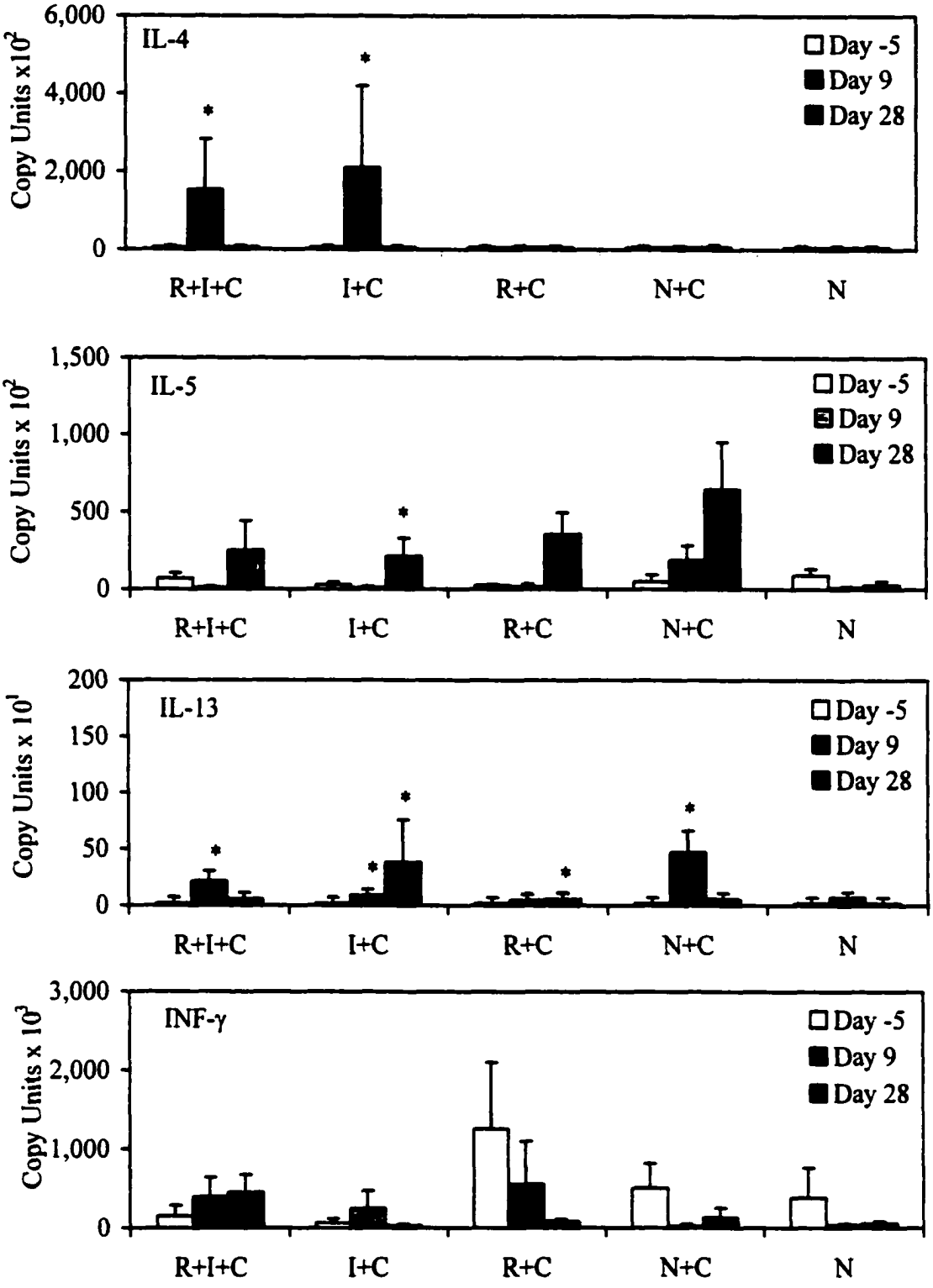

Figure 3.7. Cytokine copy units in pony peripheral blood mononuclear cells (PBMC). * Cytokine levels are significantly greater than pre-challenge (Day -5) levels. Error bars represent 1standard error of the mean.

trend towards increased IL-5 on D -5 and D 9 in the CLNC compared to other treatment groups. While the CLNC of all challenged ponies had significantly elevated IL-13 on D 9 compared to D -5, ponies in the R+I+C and I+C groups had significantly greater IL-13 levels than ponies in the other three treatment groups. The number of IL-13 copy units remained elevated above pre-challenge levels in I+C, R+C and N+C ponies through D 28. The INF-γ mRNA production was not elevated in any treatment group until D 28 and R+I+C ponies were the only group with significantly increased levels.

Additional analysis of the INF-γ response revealed that two of the four ponies in the R+C group produced 40-fold more INF-γ copy units and 18-fold fewer IL-4 copy units in the CLNC on D 9 compared to the other 2 ponies in this treatment group. More than twice as many larvae (100 verses 41) were recovered from the ponies that exhibited the high INF-γ response. These ponies also exhibited 6 more days of pyrexia and lost an average of 21 lbs compared to 16.5 lbs in the low INF-γ producers. Moreover, ponies with increased INF-γ production demonstrated a trend towards higher ICC arterial lesion scores for thickness, thrombus formation and aneurysm development (data not shown).

Analysis of PBMC cytokine mRNA levels revealed that IL-4 levels in the R+I+C and I+C groups were significantly greater on D 9 compared to pre-challenge levels and that the values were also significantly greater than the R+C, N+C and N pony groups. Only the I+C ponies had significantly elevated levels of IL-5 on D 28 compared to D -5. The IL-13 levels in PBMC were significantly elevated on D 9 and D 28 in all challenged ponies even though the overall cytokine copy units were low in all

treatment groups. The PBMC INF-γ levels were not significantly elevated in any of the five treatment groups, however, there was a trend towards increased INF-γ on D -5 in R+C ponies that declined following challenge.

Discussion

Previous studies have shown that Ribi-SAWA immunization increases Th1 cytokines whereas IrrL3 vaccination induces Th2 cytokines in ponies following challenge with *S. vulgaris* (Horohov et al., unpublished observations). The results to date suggest that Th2 cytokines are important, if not essential, in the protective immune response to *S. vulgaris* (Swiderski et al., 1999a; 1999b). Observations during the current experiment support this contention. These findings are consistent with several murine models that show that protection against gastrointestinal and tissue-migrating helminths is dependent upon dominate Th2 cytokine responses (Finkelman et al., 1997). Furthermore, the three hallmarks of nematode infection i.e., (eosinophils, mast cells and IgE), are clearly controlled by cytokines of the Th2 T-cell subset (Else and Finkelman, 1998). Beyond humans and rodent models, however, there is no definitive proof for the existence of Th1/Th2 T-cell subsets. Studies in sheep have correlated reduced Th1 cytokines with protection against *Trichostrongylus colubriformis* (McClure et al., 1995) and increased Th2 cytokines with protection against *Haemonchus contortus* (Gill et al., 2000). In contrast, cattle protected against *Ostertagia ostertagi* show a positive correlation between reduced IL-4 mRNA levels and the number of worms recovered at necropsy (Almeria et al., 1998). The studies performed in *O. ostertagi* infected cattle demonstrate that the immune response in cattle and potentially other animals is unique to that observed in murine models. The later finding illustrates the importance of

performing immunological studies of parasite infections in the natural host-parasite system. The majority of information known regarding equine immunity to nematodes has been gathered from the *S. vulgaris* helminth-naïve pony model (Klei, 2000).

In this study, an attempt was made to investigate the role of Th1 cytokines on the induction of Th2 cytokines. Current understanding of T-cell activity has emphasized the importance of early events in determining the outcome of the immune response. Polarization of T-helper subsets is heavily influenced by the local cytokine environment in which they develop (Reiner and Locksley, 1995). Production of IL-12 and INF-γ favor Th1 activation, whereas IL-4 is required for Th2 development (Mosmann and Coffman, 1989). The reduced level of INF-γ seen in most GI nematode infections is consistent with this interpretation. Experiments using *Nippostrongylus brasiliensis* show that administration of recombinant INF-γ (rINF-γ) or rIL-12 before infection can prevent the development of a Th2-associated protective immune response (Leonard, 1999). In the absence of reagents or technology to specifically eliminate Th2 cytokine production in equids, an alternative approach was used to further define the role of T-cells in the protective equine immune response to *S. vulgaris*. The hypothesis tested was that Ribi-SAWA vaccination would induce a milieu of Th1 cytokines that would down-regulate the IrrL3 vaccine-induced Th2 cytokine response and render animals susceptible to challenge infection.

Immunization with Ribi-SAWA did induce an early INF-γ response in PBMC as well as additional changes in cytokine, cellular, and clinical responses. However, IrrL3 vaccination of R+I+C ponies still favored the production of Th2 cytokines and provided protection against challenge. Concurrent immunization with Ribi-SAWA and IrrL3 did

induce an exacerbated inflammatory response following vaccination in 2 of 3 ponies that was clinically expressed as pyrexia and depression. Enhanced inflammatory mediators were likely elicited due to intensified antigenic stimulation following administration of the IrrL3 vaccine and the second Ribi-SAWA vaccine. In retrospect, cytokine analysis at this time following vaccination would have been useful for determining the Th1/Th2 cytokine profile. If Ribi-SAWA did elicit an initial Th1 response to vaccination, this response was subsequently down regulated by IrrL3 administration.

Following challenge, the clinical response in R+I+C animals was minimal and the number of days they exhibited anorexia, depression, and colic was not significantly different from the I+C or N controls. In contrast to these findings, Monahan et al. (1994) reported that Ribi-SAWA vaccinated ponies experienced an exacerbation of clinical signs and increased arterial lesions following challenge. Since the reported exacerbated response in Ribi-SAWA recipients was not due to a difference in worm burdens, it was suggested that parenteral immunization with soluble antigens sensitized these recipients and heightened their responses to challenge. Recent observations indicated that Ribi-SAWA vaccination induced an elevated INF-γ response in the CLNC (Horohov et al., unpublished information). It was hypothesized that this increase in INF-γ exacerbated the clinical and pathologic response in Ribi-SAWA vaccinates. In the current study, Ribi-SAWA immunization was not uniformly effective in inducing a mucosal Th1 cytokine response. Two of four ponies in the R+C group did experience elevated CLNC INF-γ levels nine days following challenge. Consistent with the previous report by Monahan et al. (1994), these two ponies had an increased larval

recovery, a worsened clinical response and more severe arterial lesions. The IL-4 mRNA levels were also reduced 18-fold indicating a down-regulation in the Th2 immune response. If cytokine levels had been measured at additional time points prior to D -5, a more polarized Th1 cytokine response may have been demonstrated in the PBMC and/or CLNC of the other Ribi-SAWA vaccinated ponies. It is also possible that INF-γ protein was present within the blood or cecal lymph nodes during the challenge period even though the cells collected were not producing INF-γ mRNA. The late production of INF-γ (D 28) in the CLNC of all challenged animals was likely a result of inflammation and phagocytosis of dead larvae and not due to vaccination.

In addition to Th2 cytokines, IrrL3-induced protection is also associated with an anamnestic eosinophilia (Monahan et al., 1994; Swiderski et al., 1999a). Historically, eosinophils have been recognized as a distinctive feature of the immune response mounted against helminth infections (Behm and Ovington, 2000). The demonstration that eosinophils could kill the early schistosomula stage of *Schistosoma mansoni* in vitro sparked research into their role as major effector cells in resistance to helminth infections (Meeusen and Balic, 2000). In vivo, degranulated eosinophils aggregate in the locality of helminths and eosinophil-derived proteins can be found on the surfaces of dead and damaged larvae (Pritchard et al., 1997; Behm and Ovington, 2000).

The data from this report and earlier studies support a role for eosinophils in protective immunity to *S. vulgaris* (Monahan et al., 1994; Swiderski et al., 1999a). As shown previously, ponies vaccinated with IrrL3 develop an anamnestic eosinophilia on D 9 that continues to rise through D 28 (Monahan et al., 1994). Although R+I+C ponies also demonstrated an anamnestic eosinophilia, their response was reduced and

delayed compared to I+C animals. Ponies that were vaccinated solely with Ribi-SAWA did not demonstrate an eosinophilia until D 28, and their levels were delayed and diminished compared to the N+C ponies. These results indicate that solo Ribi-SAWA immunization reduced peripheral eosinophils, however, when IrrL3 was concurrently administered, the eosinophil response was partially restored and correlated with protection. The kinetics of the eosinophil response in I+C, R+C, and N+C ponies were similar to previous reports (Monahan et al., 1994) and correlated with an increase in IL-5 production.

IL-5 is an important factor that promotes the growth, differentiation, activation and release of human and murine eosinophils from the bone marrow (reviewed in Finkelman et al., 1991; Leonard 1999). Although helminth infections are correlated with an increase in serum IL-5, eosinophilia, and eosinophil infiltration around the invading parasite, a direct role for IL-5 in parasite killing remains elusive. Interleukin-5 deficient mice fail to develop blood or tissue eosinophilia following parasitic infection, but the subsequent effect on worm burden varies with the specific parasite studied. For example, there is no correlation between IL-5 and eosinophils with the survival, growth, and fecundity of *Toxocara canis* (Sugane et al., 1996), *Trichuris muris* (Betts and Else, 1999), *Heligomosimoides polygyrus*, or *N. brasiliensis* (Leonard et al., 1999). Eosinophils, however, have been suggested to play a role in protection against tissue migrating helminths such as *Strongyloides spp.* (Grove et al., 1986; Korenaga et al., 1991), *Angiostrongylus cantonensis* (Sasaki et al., 1993), and *H. contortus* (Rainbird et al., 1998).

In this study, elevated CLNC IL-5 levels in IrrL3 vaccinates on D -5, D 9 and D 28 correlated with an early increase in blood eosinophils and enhanced eosinophil infiltration of the cecum. In comparison to I+C animals, the R+I+C ponies showed a trend toward reduced CLNC IL-5 levels and peripheral eosinophils. In both treatment groups, however, the CLNC IL-5 response appeared to be compartmentalized since PBMC IL-5 levels did not increase until D 28. In contrast, previous studies have reported similar levels of IL-5 in PBMC and CLNC on D 9 (Swiderski et al., 1998b). The delayed (D 28) CLNC IL-5 response in the R+C and N+C ponies correlated with their diminished peripheral and cecal eosinophil counts.

Although peripheral eosinophils in the R+I+C ponies were reduced compared to I+C animals, they had similar numbers of cecal eosinophils in the submucosa and lamina propria on D 28. In both groups many of the eosinophils within the inflamed areas appeared to be degranulated since there was disruption of the cell membrane and scattering of eosinophil granules. In contrast, the R+C ponies had significantly fewer eosinophils in the submucosa and lamina propria, and of those eosinophils present, none showed evidence of degranulation. Since high numbers of eosinophils within the cecum of protected (R+I+C and I+C) ponies appeared to be degranulated, these findings are suggestive of eosinophil activation and a potential important role in larval killing within the intestinal submucosa.

Histologic analysis of the liver sections reveled that the R+I+C animals had a greater infiltration of eosinophils and mononuclear cells within the hepatic periportal regions. The eosinophils in R+I+C ponies may have been sequestered within the periportal regions of the liver resulting in a reduced peripheral eosinophilia compared to

I+C animals. Although I+C animals also demonstrated periportal inflammation, the response was not as severe as that seen in the R+I+C ponies. Periportal inflammation has been previously reported in IrrL3 vaccinates and was suggested to result from the protective immune response generated within the intestinal submucuosa and the resulting clearance of larval antigens via the portal system (Monahan et al., 1994).

The enhanced inflammatory response within the R+I+C ponies may have resulted from the additive effect of eosinophil degranulation and pro-inflammatory cytokine release from the dual immunization regime. In vitro, the active component of Ribi adjuvant (monophosphoryl lipid A) has been shown to induce IL-1 and TNF-α in murine and human macrophages as well as to induce INF-γ and IL-2 in human peritoneal lymphocytes (Ulrich and Myers, 1995). In vivo, Ribi adjuvant increased the INF-γ response in mice vaccinated with influenza virus (Mbawuike et al., 1996). In the current study, although increased INF-γ levels were not measured in the dual immunized group, it is possible that Ribi-SAWA vaccination induced additional inflammatory mediators such as IL-1 and TNF-α that lead to the exacerbation of the periportal inflammatory response. It is also possible that the increased exposure to *S. vulgaris* antigens in R+I+C ponies lead to accelerated larval killing within the intestinal submucosa and a more severe periportal inflammatory reaction. Although not significant, animals in the R+I+C group had a lower larval recovery rate and diminished arterial lesion scores.

In the *S. vulgaris* model, a relationship has not been established between IL-4 levels, increased cecal mast cell numbers, and protection. Whereas IL-4 was elevated in both protected and nonprotected animals, the number of submucosal mast cells did not

increase. However, the lack of mast cells visualized within the lamina propria may have been an artifact of the tissue fixation technique utilized and not the immunization regimes. It is possible that, if tissues had been fixed with Carnoys solution instead of 10% formalin, a difference in mast cell number might have been observed in the lamina propria. However, since there was no significant difference in submucosal mast cells in this study or in previous reports (Swiderski et al., 1999b; Horohov et al., unpublished observations) these results argue against a role for mast cells and IL-4 in protection against *S. vulgaris*. Similarly, in other host-parasite systems it has been demonstrated that IL-5 can act independently of IL-4 in providing protection against helminths. For example, murine studies have shown that immunity to *Onchocerca lienalis* is dependent upon IL-5, but not IL-4 in eliciting an eosinophil mediated parasite clearance (Hogarth et al., 1998).

Ribi-SAWA immunization also induced alterations in an additional Th2 cytokine, IL-13. There was a trend toward increased CLNC IL-13 levels in R+I+C ponies on D 9 compared to the I+C animals. Although the IL-13 levels in ponies vaccinated only with Ribi-SAWA increased following challenge, their levels were significantly lower than those in R+I+C and I+C ponies. This response appeared to be compartmentalized since there was minimal IL-13 production in the PBMC following challenge. Because the elevated IL-13 levels were measured in protected animals, these results suggest that increased IL-13 may be associated with immunity to *S. vulgaris*. The specific role for IL-13 in the *S. vulgaris* model is unknown. In mice, IL-13 has been shown to be a key cytokine in mediating protection against several gastrointestinal nematodes including *N. brasiliensis* and *T. muris* (reviewed by Finkelman et al., 1999).

IL-13 shares many biologic functions with IL-4 due to a common α-chain receptor and signaling through the signal transducer and activator of transcription (STAT) 6-dependent pathway (reviewed by McKenzie, 2000). Some cells are more responsive to IL-4 or IL-13 since relative receptor chain expression varies with cell type (Finkelman et al., 1999). It is currently not known which cell phenotypes in equids are responsible for IL-13 production. In mice, Th2 cells secrete IL-13, whereas in humans both Th1 and Th2 cells produce IL-13 (Finkelman et al., 1999; Zurawski and de Vries, 1994).

Interleukin-18, originally named INF-γ inducing factor, is an important cytokine in the induction of proinflammatory responses in the gut. A novel role for IL-18 was recently defined in induction of Th1 responses through down-regulation of IL-13. It was further shown that the regulatory role of IL-18 was independent of IL-12 and INF-γ (Helmby et al., 2001). In the *S. vulgaris* model, INF-γ is not produced until 28 days after infection and therefore additional cytokines such as IL-18 may be acting to down-regulate a potentially protective IL-13 response and lead to chronic infections in the non-IrrL3 vaccinated ponies.

The results of this study further characterize the protective cellular immune response to *S. vulgaris*. The increase in IL-5 production and corresponding increase in blood and tissue eosinophil levels during the course of parasite elimination in R+I+C and I+C ponies argues for a central role of IL-5 and eosinophils in protective immunity. Similar findings have been reported for a range of tissue-dwelling nematodes in rodents (Hogarth and Bianco, 1999). In addition to IL-5, several other factors that were not measured in the current study have also been shown to be involved in eosinophil mobilization and migration. Some of these factors include adhesion molecules such as

α4β7, ICAM-1, and VCAM-1; additional cytokines such as IL-1, IL-3, IL-4, IL-13, GM-CSF; and local chemoattractants such as eotaxin, platelet activating factor, and leukotriene B_4 to name a few (Rothenberg et al., 2001). The role of these agents in equine eosinophil activation and accumulation are currently unknown, however, they may contribute to an eosinophil-mediated protective immune response. Although IL-4 mRNA was increased in both protected and nonprotected ponies, IL-13 mRNA was induced only in protected animals and may argue for its role in immunity. In addition to characterizing the protective immune mechanisms against *S. vulgaris*, this investigation also attempted to address the roie of Th1 cytokines on the induction and regulation of equine immunity. Contrary to the original hypothesis, the Th1 response induced by Ribi-SAWA sensitization was not sufficient to alter protection. It is possible that immunization with a more specific Th1 inducing agent, such as immunostimulatory oligodeoxynucleotides (CpG) (Chu et al., 1997), may provide a stronger stimulation and down-regulate the Th2 response. Future studies are directed toward defining the potential role of equine T-cells and eosinophils in protective immunity. In an effort to elucidate the role of T-cells and eosinophils in protective immunity, these studies will focus on the use of anti-IL-5 and anti-T-cell antibodies to ablate the respective cell populations and to study the resulting altered immune responses.

CHAPTER 4
ANTIBODY RESPONSES TO *STRONGYLUS VULGARIS* INFECTION IN PARASITE-FREE PONIES FOLLOWING DIFFERENT IMMUNIZATION PROTOCOLS

Introduction

Strongylus vulgaris is a parasitic nematode of equids that undergoes extensive larval migration in the intestinal arteries prior to adult establishment in the cecum and large intestine (reviewed in Ogbourne and Duncan, 1985). This larval migration leads to arteritis and thrombosis of the small submucosal arteries (White et al., 1985). As a result, affected ponies exhibit pyrexia, depression, anorexia, and colic all within the first 14 days of infection (Duncan and Pirie, 1972; Klei et al., 2000). In severe cases, large thrombi develop within the arterial lumen and release emboli that compromise perfusion to downstream intestinal vascular beds. The syndrome is termed verminous arteritis or thromboembolic disease. It is characterized by ischemic infarction of the small and/or large intestine resulting in severe abdominal pain, endotoxemia, and death (White et al., 1985). Although *S. vulgaris* is easily controlled in closed herds of horses that are treated regularly with macrocyclic lactones, it still remains prevalent in nontreated equine populations (Klei et al., 1992). A great deal of research has been performed in *S. vulgaris* infected equids and it has proven to be a valuable research model for studying the equine immune response to nematodes.

Exposure to irradiated *S. vulgaris* L3 significantly reduces larval burdens and prevents the development of clinical disease (Klei et al., 1982). It has been hypothesized that IrrL3 induced protection occurs at the level of the intestinal submucosa and several immune mechanisms including anti-*S. vulgaris* antibodies are likely involved. Antibodies specific for soluble adult worm antigens (SAWA) and L3

surface antigens are produced in protected animals and may play a role in eliminating migrating larvae through antibody mediated cellular cytotoxicity (ADCC) mechanisms. In vitro assays demonstrated that an anti-L3 antibody in immune sera promotes adherence of activated eosinophils but not other cell types to *S. vulgaris* L3 and results in L3 death. This antibody response has been shown to be species specific to the L3 stage of *S. vulgaris* and does not cross-react with the L3 stage of the closely related nematode, *S. edentatus* (Monahan et al., 1994). When freshly exsheathed *S. vulgaris* were cultured in vitro overnight or for 3 days, the surface antibody responses measured by indirect fluorescent antibody titer (IFAT) were enhanced to the cultured larvae (Monahan et al, 1994). Unique L3 surface proteins (Monahan et al., 1994) and soluble L3, L4, L5 and adult protein antigens (Klei, 1992) are recognized by immune serum. These findings suggest that there are shifts in soluble and surface protein production at different stages during *S. vulgaris* development. The relevance of these larval and adult antigens to protective resistance is currently unknown, however, they may represent important immune targets.

The IgG subisotype response to *S. vulgaris* SAWA following IrrL3 vaccination has been characterized. Data from recent studies revealed that pre-challenge levels of soluble adult-specific IgG(T), IgGa, and IgGb are significantly higher in the sera of IrrL3 vaccinates compared to naïve animals (Swiderski et al., 1999a). Furthermore, following challenge, the IgG(T) levels remain significantly higher in IrrL3 recipients (Swiderski et al., 1999a). Challenge with *S. vulgaris* also results in significantly increased IgA levels in both IrrL3 vaccinated and nonvaccinated ponies while elevated IgGa levels are only seen in nonvaccinates (Swiderski et al., 1999a). The significance

of the different antibody isotype and subisotype responses between IrrL3 vaccinates and nonvaccinates remains unclear since the biological function and cytokine regulation of equine subisotypes are unknown.

In contrast to IrrL3 vaccination, intramuscular immunization with soluble adult worm extracts with Ribi adjuvant (Ribi-SAWA) induced an anamnestic IgG response to both soluble adult and larval *S. vulgaris* antigens. The vaccination, however, was not protective and resulted in the exacerbation of lesions normally associated with larval migration (Monahan et al., 1994). The exacerbation of arterial lesions seen in the face of high antibody titers does not rule out a role for antibodies in protective immunity. Although antibodies to soluble adult and larval antigens were produced in Ribi-SAWA vaccinates, these ponies had reduced IgG titers to the surface of freshly exsheathed L3 and cultured L3 stages of *S. vulgaris* compared to IrrL3 vaccinates. It was hypothesized that the reduced IgG response to the L3 surface may have been attributed to the absence of a protective immune response in ponies immunized with Ribi-SAWA.

The close contact between tissue-migrating helminthes such as larval stages of *S. vulgaris* and the host immune system results in direct access of parasite antigens to host immune effector mechanisms. The objective of this study was to further characterize the protective equine antibody response to *S. vulgaris* and to measure the effect of Ribi-SAWA sensitization on IrrL3 induced antibodies. This is the first report on the specific IgG subisotype(s) directed to soluble somatic L3 and L4 antigens and to L3 surface antigens. The hypothesis was that IrrL3 vaccination would produce a distinct antibody profile to the L3 surface as well as to L3, L4 and adult somatic antigens. It was further hypothesized that concurrent Ribi-SAWA immunization would produce a

Th1 cytokine milieu and thus would alter the isotypic and IgG subisotype antibody response to *S. vulgaris* antigens.

Materials and Methods

Experimental Design

The experimental design was described in Chapter 3.

Strongylus vulgaris Larvae Preparation and L3 Culture

Strongylus vulgaris L3 were collected from Baermann sedimentation of fecal cultures from monospecifically infected donor ponies (McClure et al., 1994). Following harvest from the Baermann apparatus, larvae were washed in tap water and exsheathed with a 1.3% fresh sodium hypochlorite solution. Exsheathed larvae were washed five times with tap water to remove the shed cuticles and residual sodium hypochlorite solution. At this stage of preparation, larvae were then either stored at -70°C until used in the IFAT assays (early L3) or were placed immediately into culture (developing L3) according to previously established protocol. The developing L3 were washed once with NCTC-135 (Gibco, Grand Isle, NY) supplemented with 2.8 gL^{-1} Bacto-Peptone (Difco Laboratories, Detroit, MI), 2.25 gL^{-1} yeast extract (Difco), 2.8 gL^{-1} dextrose (Difco), antibiotics (sodium penicillin G: 400 Uml^{-1}; streptomycin sulfate: 400$\mu g\ ml^{-1}$; amphotericin B: 1$\mu g\ ml^{-1}$; Gibco, Grand Island, NY) and 50% fetal calf serum (Chapman et al., 1994). A gas mixture of 10% CO_2, 5% O_2, and 85% nitrogen was bubbled through the media and larvae prior to incubation at 37°C in 5% CO_2 (Chapman et al., 1994). Larvae were cultured for 3 days and then washed five times with PBS to remove residual media and used immediately in IFAT assays.

Soluble Antigen Preparation

Male and female adult *S. vulgaris* were collected from equine intestines and L4 were collected from cranial mesenteric artery scrapings. Parasites were washed extensively in PBS and stored at –20°C prior to use. Adult and L4 were thawed and prepared separately. Each was ground with a mortar and pestle and then subjected to repeated cycles of grinding in a glass tissue homogenizer followed by repeated cycles with a Polytron electric homogenizer (Kinematica, Switzerland). Homogenates were stirred overnight at 4°C with 2.5% aprotnin (Sigma, St. Louis, MO). Following overnight solubilization, antigen preparations were centrifuged at 10,000-x g for 30 minutes at 4°C. Supernatants were filtered through a 0.45 μm filter and protein content was determined using a commercial kit (Bio-Rad, Hercules, CA). The preparations were then aliquoted and stored at -70°C until used.

The *S. vulgaris* L3 were not of sufficient size to be disrupted by the mortar and pestle and glass tissue homogenizer, so instead L3 were homogenized for 15 minutes at 21,000 rpm with a Tissue Tearor (BioSpec, Bartlesville, OK). Further steps for preparation follow the above description for adult and L4 antigens.

Enzyme-Linked Immunosorbent Assay (ELISA)

Circulating antibody levels to adult, L4 and L3 *S. vulgaris* soluble antigens were monitored by ELISA using serum collected on days 0, 3, 9, 14, and 28 following challenge. Microtiter plates were coated overnight at 4°C with 10 μg/ml of soluble antigen. ELISAs for serum antibody levels were performed as previously described (Swiderski et al., 1999a). Coated plates were washed with PBS containing 0.05% Tween 20 in an automatic plate washer and blocked for 1 hour with PBS containing 1%

fish gelatin (PBSG). Serum samples were diluted 1:100 in blocking buffer and added to the wells in triplicate. After a 1 hour incubation at 39°C, monoclonal (Mab) anti-equine IgA, IgGa, IgGb or IgG(T) diluted 1:100 or horseradish peroxidase-conjugated anti-equine total IgG (Kirkegaard and Perry, Gaithersburg, MO) diluted 1:10,000 was added to the appropriate wells. After a second 1 hour incubation at 39°C, plates with Mabs were washed and incubated an additional hour with affinity-purified horseradish peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MO). Plates were developed with 3,3', 5,5'-tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MO) and analyzed at OD_{450}. ELISA units of each antibody isotype were determined by interpolation from a standard curve that was generated using serum from a known high responder.

Indirect Fluorescent Antibody Titer (IFAT)

Total IgG recognition of *S. vulgaris* larval surface antigens were compared among treatments using the IFAT technique with sera collected on study days 0, 3, 9, 14, and 28. On D 9 and D 14 the IgGa, IgGb and IgG(T) antibody responses were also analyzed by IFAT. These days were chosen for subisotype analysis since they were the earliest times showing elevated total IgG titers. *S. vulgaris* L3 were tested at two different stages in development: immediately as freshly exsheathed L3 (early L3), or following a 3 day in vitro culture (developing L3).

Serum was diluted 2 or 3-fold in PBS and 50 µl of each dilution was added in duplicate to Immulon I B flat-bottom microtiter plates (Dynex Tech., Chantilly, VA). Larvae were suspended in PBS at a concentration of 2 L3/µl and added to all wells in 50 µl volumes. Plates were incubated for 1 hour at 39°C and then washed three times with

PBS by gently aspirating the fluid with a multichannel pipettor. Monoclonal anti-equine IgA, IgGa, IgGb or IgG(T) (Serotec, Raleigh, NC) diluted 1:30 were added in 50 µl volumes or 25 µl fluorescein-labeled mouse anti-horse IgG, heavy and light chain specific (Kirkegaard and Perry, Gaithersburg, MO) diluted 1:15 was added to the appropriate wells. After a second, 1 hour incubation at 39°C, plates with Mabs were washed and incubated an additional hour with 25 µl fluorescein-labeled goat anti-mouse IgG, heavy and light chain specific (Kirkegaard and Perry, Gaithersburg, MO) diluted 1:50. All wells were washed five times after the final incubation and examined on a Nikon Diaphot-TMD inverted microscope equipped with epifluorescence (Nippon Kogaku, Tokyo, Japan). The end-point for the IFAT was determined at the last dilution that the larvae were positive for fluorescence. Serum from a parasite-free pony served as a negative control and serum from a known high responder was used as a positive control. Additional controls included; wells containing larvae, positive serum, and secondary antibody (fluorescein-labeled goat anti-mouse IgG), and wells containing larvae and fluorescein-labeled anti-equine antibody only.

Statistics

Statistical analysis was performed using Sigma Stat software (Chicago, IL). ELISA units were log transformed to normalize the data. The log transformed ELISA units and IFAT titers were analyzed by a one way ANOVA. When values were significant, all pairwise comparisons were analyzed using a Student-Newman-Keuls test. ELISA units were also subjected to a repeated measures one way ANOVA. A T-test was used to compare Day 9 and Day 14 IFAT titers. Differences were considered significant at $P < 0.05$.

Results

ELISA

Total IgG levels to all soluble antigens in R+I+C ponies were consistently higher than those of R+C ponies at all times following challenge with *S. vulgaris* (Figure 4.1). Although total IgG levels were higher in R+I+C ponies, both R+I+C and R+C groups lacked a demonstrable anamnestic response. In contrast, the I+C ponies exhibited an anamnestic total IgG response to L4 and L3 antigens and levels were significantly increased on D 14 and D 28 compared to D 0 values. Total IgG levels in N+C animals did not increase significantly until D 28.

The R+I+C, I+C and R+C ponies had detectable IgGa levels to SAWA on D 0 that did not change significantly after administration of the challenge infection (Figure 4.2). The IgGa response to L4 and L3 antigens, however, did increase significantly in I+C ponies on D 14 and D 28. In comparison, N+C animals had significantly increased levels on D 28 only. Furthermore, the L3-specific IgGa levels were similar in both I+C and N+C animals on D 28 and there was a strong trend toward higher IgGa levels in these animals compared to ponies in the other treatment groups.

The IgGb response in R+I+C ponies increased in an anamnestic fashion to SAWA with significant increases occurring on days 9, 14 and 28 compared to day 0 levels (Figure 4.3). Although I+C animals also had significantly increased SAWA-specific IgGb on D 14 and D 28, there was a trend toward reduced IgGb levels compared to R+I+C animals to all soluble worm antigens except to L3 antigens on D 28. The IgGb response in R+C and N+C ponies was minimal except for a significant increase in N+C ponies to L3 antigens D 28.

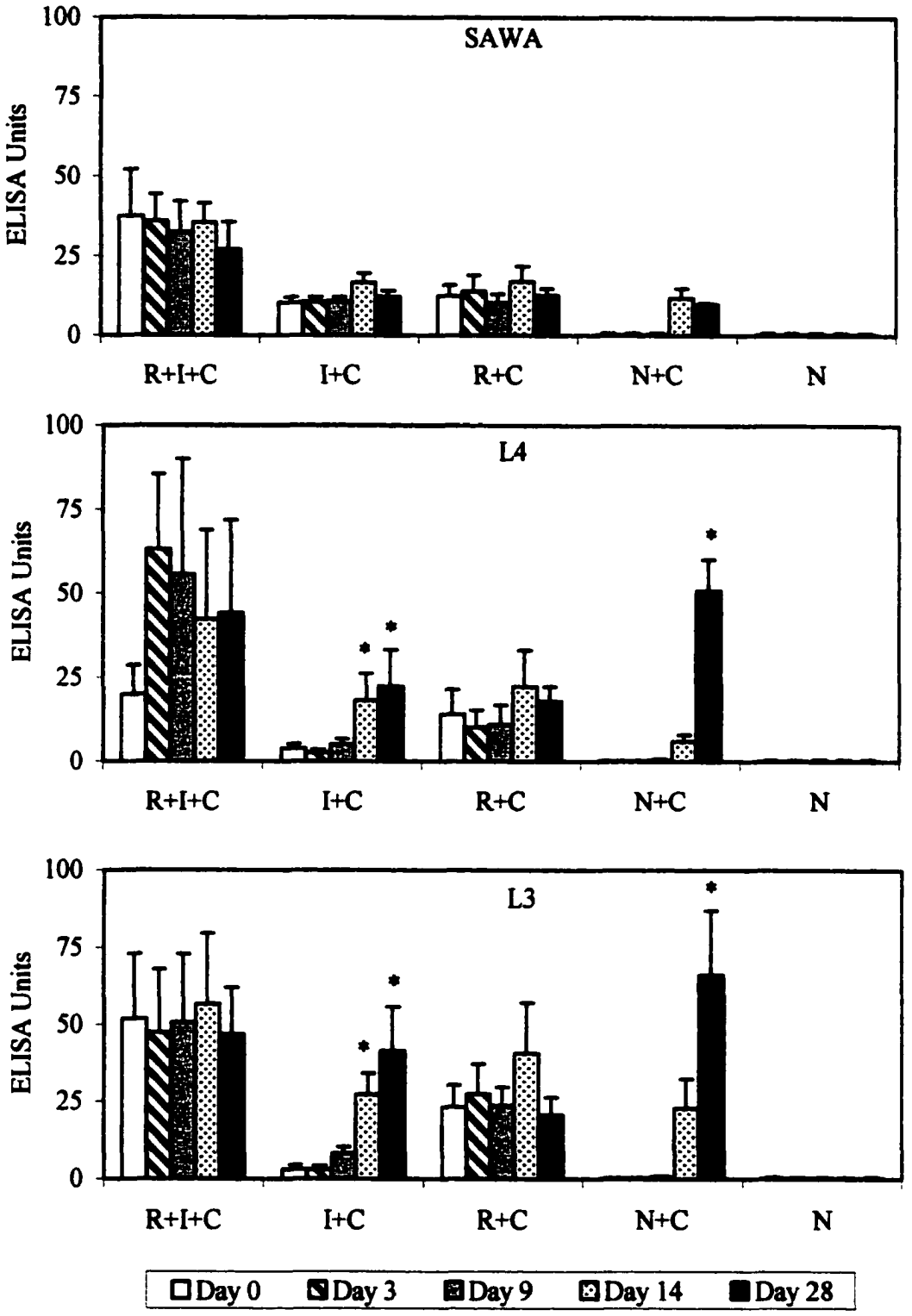

Figure 4.1. Total IgG response in ponies to *S. vulgaris* soluble adult worm antigen (SAWA) (top panel) L4 soluble antigen (middle panel) and L3 soluble antigen (bottom panel). *ELISA units are significantly greater than day 0 levels. Error bars represent 1 standard error of the mean.

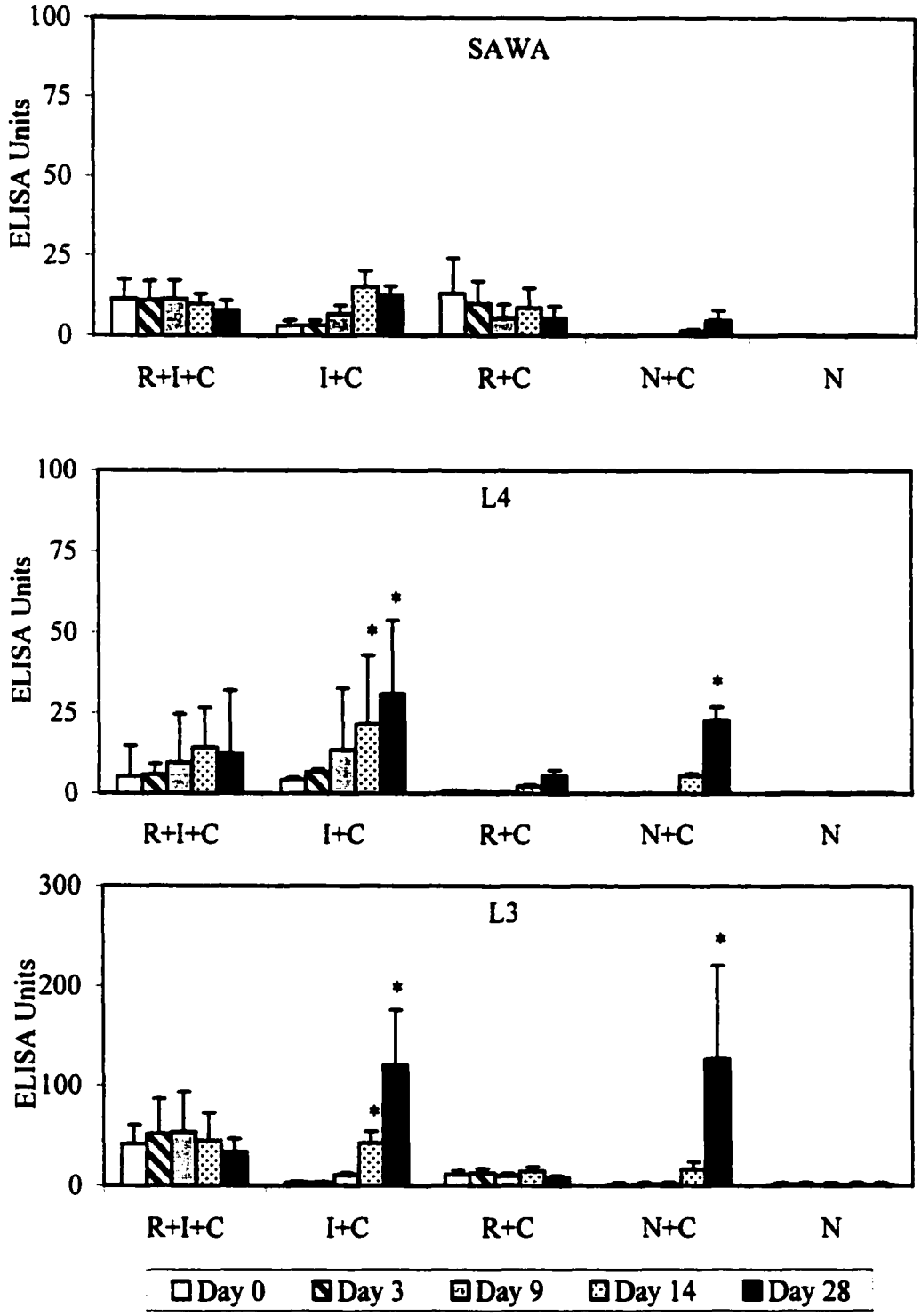

Figure 4.2. IgGa response in ponies to *S. vulgaris* soluble adult worm antigen (SAWA) (top panel) L4 soluble antigen (middle panel) and L3 soluble antigen (bottom panel). *ELISA units are significantly greater than day 0 levels. Error bars represent 1 standard error of the mean.

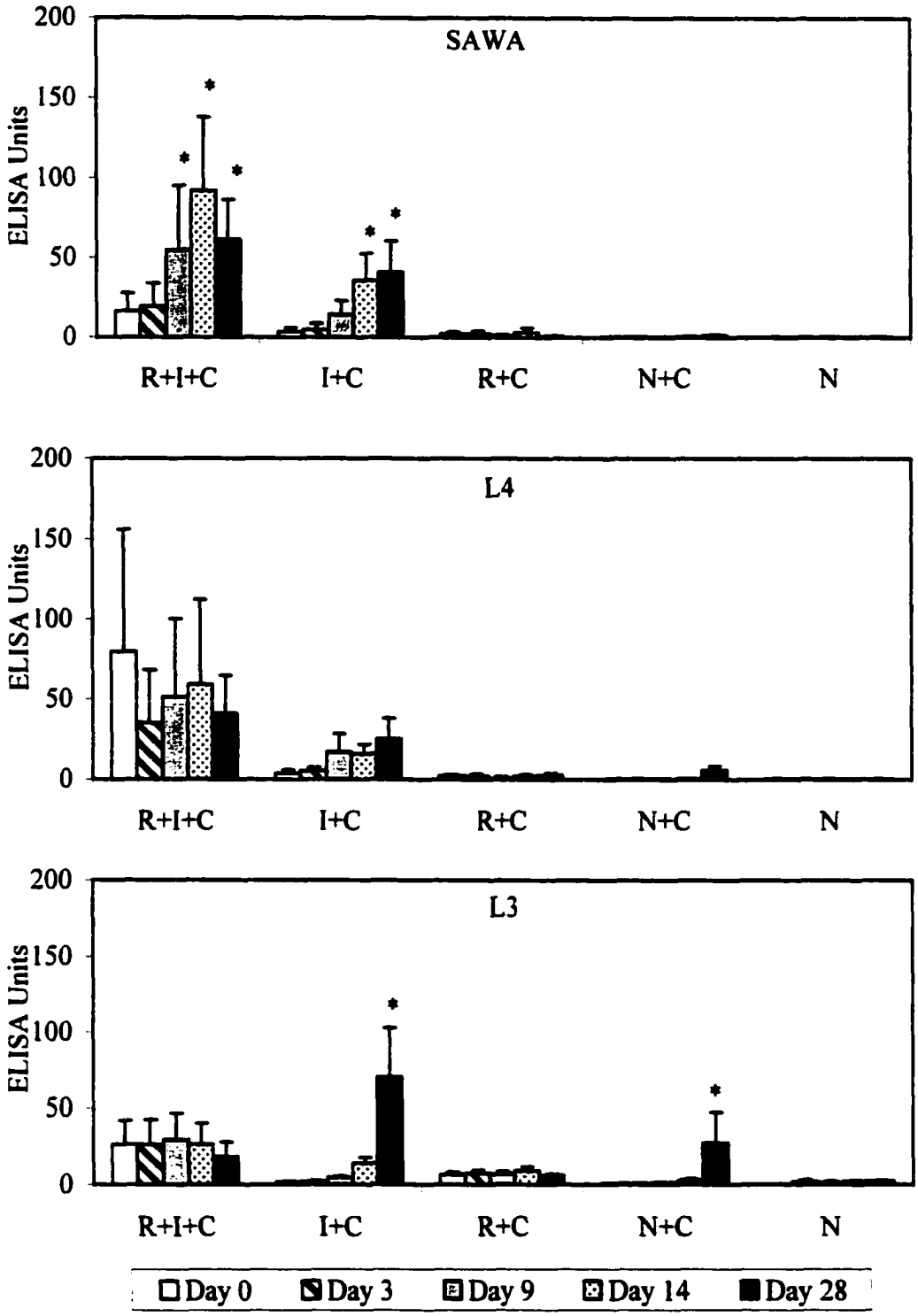

Figure 4.3. IgGb response in ponies to *S. vulgaris* soluble adult worm antigen (SAWA) (top panel) L4 soluble antigen (middle panel) and L3 soluble antigen (bottom panel). *ELISA units are significantly greater than day 0 levels. Error bars represent 1 standard error of the mean.

The I+C ponies displayed an anamnestic IgG(T) response to all soluble worm antigens and levels were significantly elevated on days 9, 14, and 28 to L4 antigens, and on days 14 and 28 to SAWA and L3 antigens (Figure 4.4). In comparison, R+I+C ponies did not exhibit an anamnestic IgG(T) response to any soluble worm antigens and had a trend towards reduced SAWA and L4-specific IgG(T) levels compared to I+C animals. The L3-specific IgG(T) response in R+I+C ponies, however, was elevated in comparison to I+C animals early in the challenge period until D 28, when the I+C ponies had a significant increase in production. There was minimal IgG(T) made in R+C and N+C ponies until D 28, when the N+C animals exhibited significantly increased levels to L3 antigens.

The R+I+C ponies produced similar levels of IgA to all soluble worm antigens at all time points measured (Figure 4.5). As seen with IgG(T), the I+C ponies exhibited an anamnestic IgA response to all soluble worm antigens and levels were significantly higher on days 9, 14, and 28 to L4 antigens, on days 14 and 28 to SAWA and on day 28 to L3 antigens. The R+C ponies produced minimal IgA to larval antigens while N+C ponies had significantly elevated levels to L3 antigens on D 28.

IFAT

Total IgG titers directed to the surface of early and developing *S. vulgaris* L3 were measured on days 0, 3, 9, 14, and 28 (Figure 4.6). Surface specific antibodies were minimal in all treatment groups until D 9 when titers in R+I+C and I+C animals begin to elevate. Compared to the other treatment groups, ponies vaccinated with IrrL3 displayed the highest total IgG titers on D 14 and D 28 to both early and developing L3 although these differences were not significant. The N+C ponies did not develop a

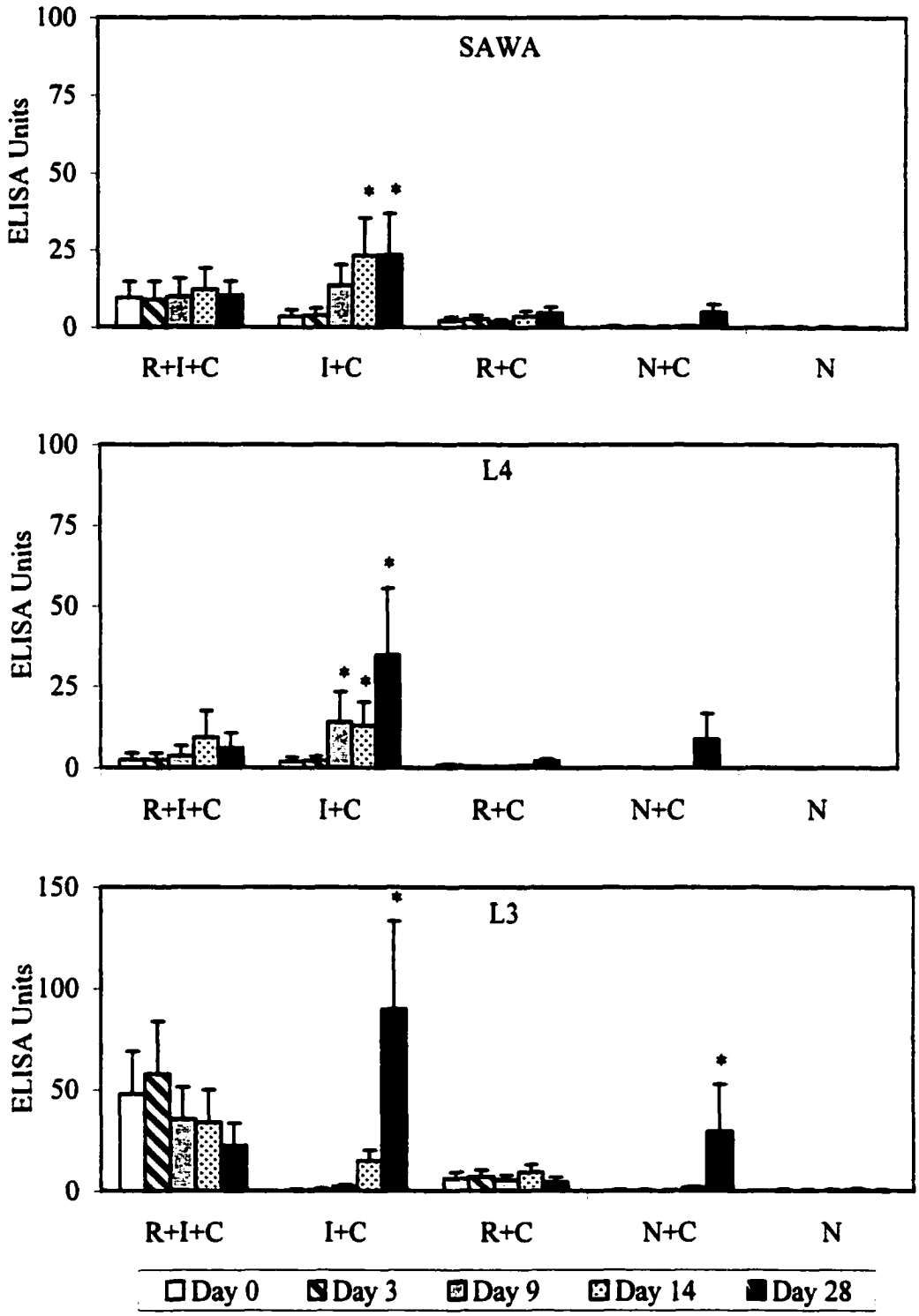

Figure 4.4. IgG(T) response in ponies to *S. vulgaris* soluble adult worm antigen (SAWA) (top panel) L4 soluble antigen (middle panel) and L3 soluble antigen (bottom panel). *ELISA units are significantly greater than day 0 levels. Error bars represent 1 standard error of the mean.

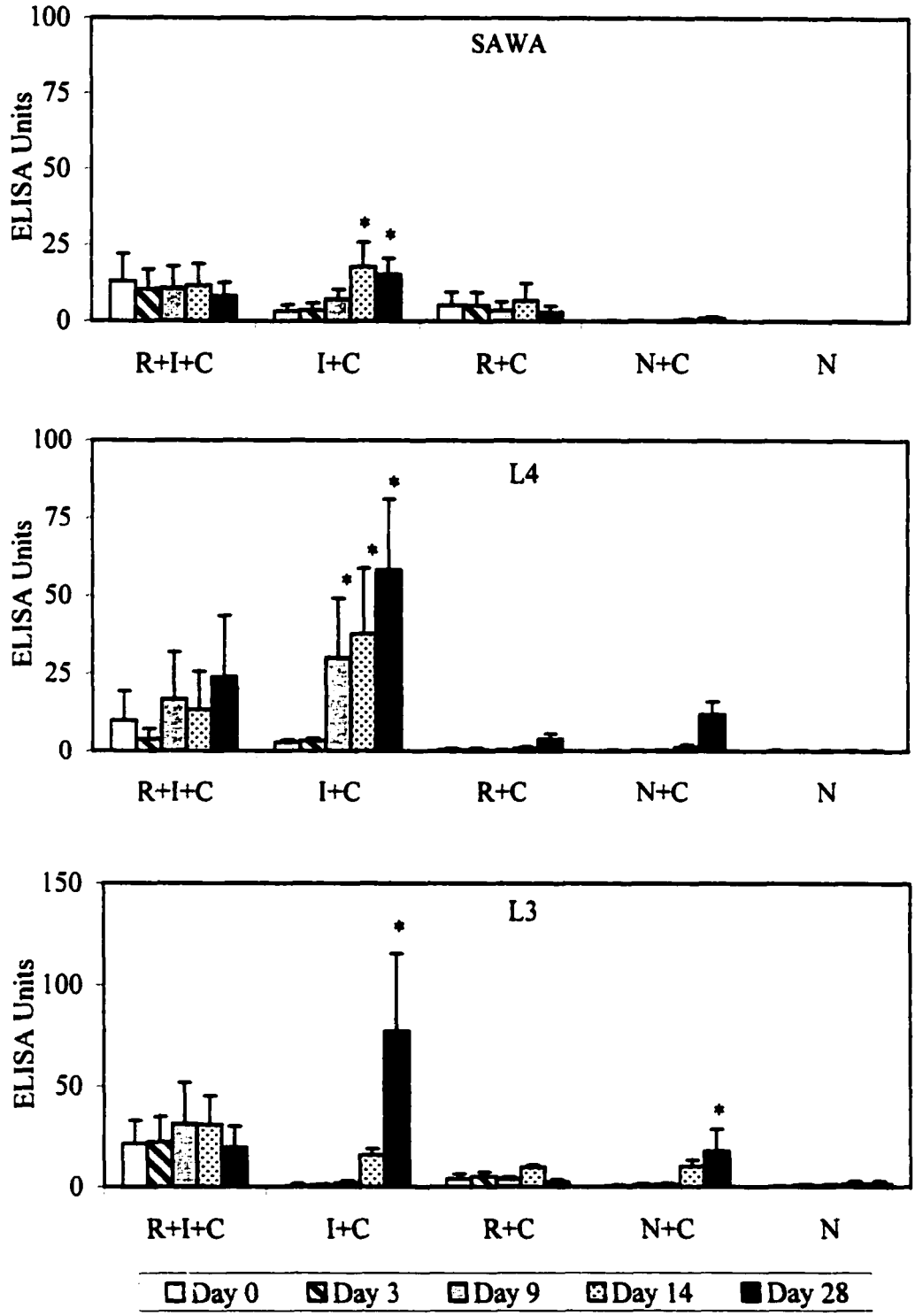

Figure 4.5. IgA response in ponies to *S. vulgaris* soluble adult worm antigen (SAWA) (top panel) L4 soluble antigen (middle panel) and L3 soluble antigen (bottom panel). *ELISA units are significantly greater than day 0 levels. Error bars represent 1 standard error of the mean.

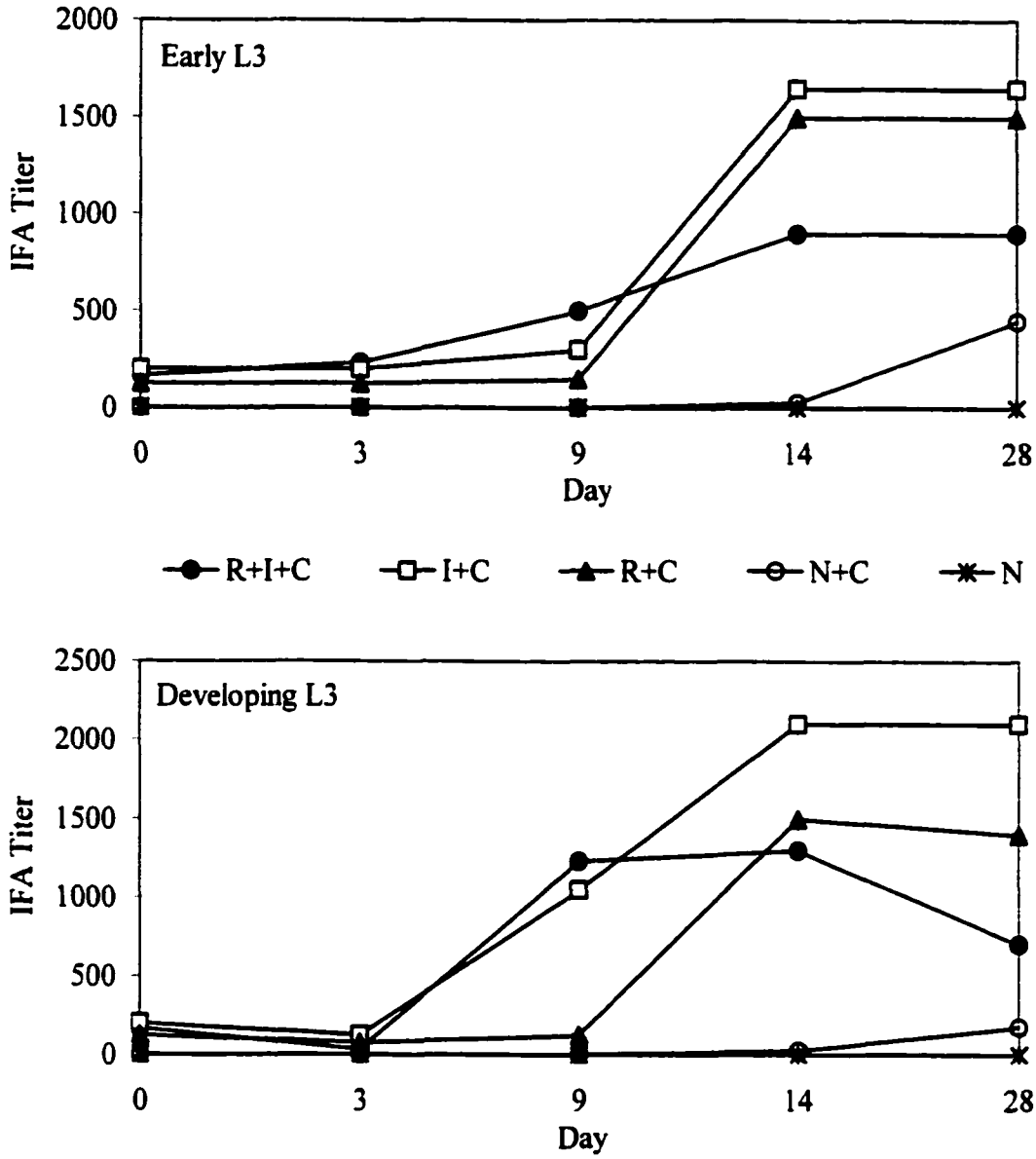

Figure 4.6. Indirect fluorescent antibody titer to early *S. vulgaris* L3 (top panel) and to developing L3 (bottom panel). Early L3 were exsheathed and then used in the assay whereas developing L3 were cultured in vitro for 3 days prior to use.

detectable L3 surface antibody response until D 28 and the highest response was to the early L3 stage. The L3 surface specific IgG subisotype responses were measured on sera collected on D 9 and D 14 since these were the earliest days that the L3 total IgG levels were elevated.

The R+I+C and I+C ponies demonstrated a trend toward elevated IgGa, IgGb, and IgG(T) levels to early and developing L3 on D 9 compared to ponies in the other treatment groups (Figure 4.7). These ponies also exhibited a trend toward increased IgGa, IgGb and IgG(T) on D 14 compared to D 9. The I+C animals appeared to have higher IgGa and IgG(T) titers to developing L3 on D 14, although the values were not significantly greater than the ponies in the other four treatment groups. Minimal levels of IgGa, IgGb, and IgG(T) were detected in R+C ponies on D 9. However, on D14, R+C ponies had significant increases in IgGa to early and developing L3, and significant increases in IgGb and IgG(T) to developing larvae. The N+C ponies did not produce detectable levels of peripheral antibodies to L3 surface antigens on D 9 or D 14.

Discussion

Previous observations suggest that antibodies generated following IrrL3 vaccination and *S. vulgaris* challenge are likely important components of the protective immune response (Monahan et al., 1994; Klei, 2000). When compared to Ribi-SAWA vaccinates, which were not protected against challenge infection, IrrL3 vaccinates had higher pre-challenge and 2 week post-challenge total IgG antibody titers to *S. vulgaris* L3 surface antigens (Monahan et al., 1994). However, immunization with either protective IrrL3 or nonprotective Ribi-SAWA, resulted in increased anti-SAWA

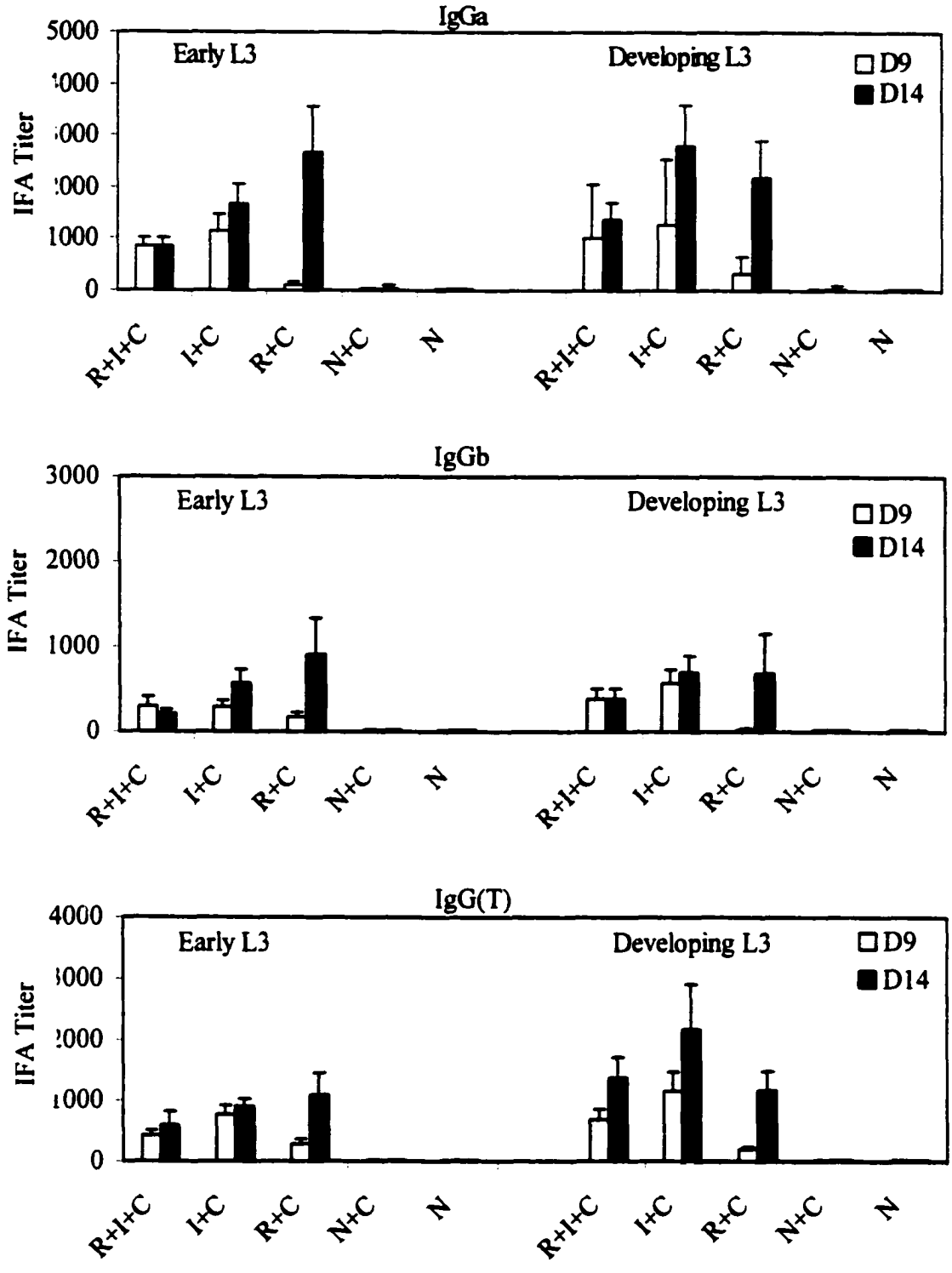

Figure 4.7. IgG subisotype indirect fluorescent antibody titer to early and developing *S. vulgaris* L3 on day 9 and day 14 post-challenge. Error bars represent 1 standard error of the mean.

specific total IgG (Monahan et al., 1994). Consistent with earlier investigations (Monahan et al., 1994), IrrL3 vaccination and Ribi-SAWA immunization in the present study resulted in increased anti-SAWA total IgG. The total IgG response to SAWA, L4 and L3 soluble antigens were also enhanced when ponies were immunized with both Ribi-SAWA and IrrL3 (R+I+C). Although both R+I+C and I+C ponies were protected against the challenge infection, there were differences between these groups in their IgG subisotype responses to adult, L4 and L3 soluble antigens.

Analysis of IgG subisotypes revealed that only IrrL3 vaccinates (R+I+C and I+C) demonstrated increased SAWA-specific IgG(T) levels. In addition, the I+C ponies produced an early IgG(T) response to soluble L4 antigens and R+I+C ponies had an early response to L3 antigens. These data are consistent with earlier studies that showed IgG(T) was the principle component in heightened β-globulin concentrations in *S. vulgaris* infected horses (Patton et al., 1978). Beyond *S. vulgaris*, further evidence suggests that the IgG(T) subisotype is induced by other helminth antigens. For example, ponies infected with a mixed cyathostome population had increased IgG(T) antibodies to SAWA following either natural pasture exposure or experimental challenge (Klei et al., unpublished observations). Similarly, horses infected with *Anoplocephala perfoliata* exhibited a marked IgG(T) response to specific excretory/secretory proteins that was strongly correlated with parasite infection intensity (Proudman and Trees, 1996). Despite this evidence for a helminth-induced IgG(T) response, an association between IgG(T) and protective resistance to *S. vulgaris* remains strictly corollary.

When Ribi-SAWA was administered along with IrrL3 (R+I+C), an elevated IgGb response was induced to SAWA, L4 and L3 antigens. The increased IgGb production may have resulted from an altered cytokine response in these animals. Ribi adjuvant has been shown to induce a biased Th1 cytokine response in murine models (reviewed in Ulrich and Myers, 1995). Correlating with the induction of a Th1 cytokine response, Ribi adjuvant also increases concentration of the murine Th1 antibodies IgG2a and IgG2b to soluble protein antigen administration (Ulrich and Myers, 1995). As reported in Chapter 3, Ribi-SAWA did induce a small trend toward increased INF-γ production in PBMCs following challenge. This INF-γ response or perhaps additional unmeasured cytokines induced by the combination of Ribi-SAWA and IrrL3 may have lead to the elevated IgGb response in R+I+C animals. Since an increased IgGb was not seen in ponies vaccinated only with Ribi-SAWA, it appears that the dual immunization regime was required for this enhanced response. The absence of elevated IgGb levels in I+C animals suggests that this subisotype was not essential for protection and may be induced by Th1 cytokines.

Animals vaccinated in all ways (R+I+C, I+C and R+C) showed similar levels of IgGa and IgA to SAWA at all times examined. Whereas the I+C ponies' IgGa and IgA responses increased significantly to L3 and L4 soluble antigens on D 14, the IgGa and IgA levels in R+I+C ponies remained consistently elevated and did not change throughout the challenge period. The increased IgG(T), IgGa and IgA responses to L4 and L3 soluble antigens following challenge in protected (R+I+C and I+C) groups were correlated with the elevated Th2 cytokine response (IL-4, IL-5, and IL-13) seen in these animals (Chapter 3). These observations suggest that *S. vulgaris* induced IgG(T), IgGa

and IgA antibodies may be regulated by Th2 cytokines. As previously noted, IgG(T) production is associated with helminth infections, however, IgGa and IgA are more often associated with Th1 responses to bacterial and viral challenges. For example, horses infected with *Streptococcus equi* (Sheoran et al., 1997) or influenza virus (reviewed by Horohov, 2000) produced elevated antigen-specific IgGa, IgGb, and IgA. Induction of IgGa and IgA therefore, are not clearly associated with either Th1 or Th2 type responses. Experiments in murine models have shown that immunoglobulin class switching is under the control of T-cell cytokines and specific Th1 and Th2 cytokines favor certain isotype and subisotype production by B-cells (Schultz and Coffman, 1991). Antibody production, however, is not dependent exclusively on one cytokine. One or more cytokines functions to stimulate immunoglobulin switching and additional cytokines regulate antibody production. There is redundancy, however, in the cytokines involved in B-cell activation and antibody secretion as shown in studies performed in cytokine knock-out mice (DeFranco, 1999). The cytokine regulation of equine isotype and IgG subisotype induction is currently unknown.

An anamnestic total IgG, IgGa, IgG(T), and IgA antibody response to L3 and L4 soluble antigens was observed in I+C ponies, but not in R+I+C animals. Instead, R+I+C ponies demonstrated an early elevated antibody response that did not increase following challenge. This response was unique to the R+I+C animals and was not observed in ponies in the other treatment groups. This suggests that vaccination with both Ribi-SAWA and IrrL3 resulted in a prolonged anti-*S. vulgaris* antibody response following immunization. Although 6 weeks separated vaccination and challenge, the antibodies in R+I+C ponies remained elevated and an anamnestic increase was not

demonstrated. Therefore, a sustained inflammatory response likely supported prolonged B-cell division within lymphoid germinal centers and obscured detection of a memory response. Since antibodies were not measured during the pre-challenge period, it is not known which antibody isotypes were produced following vaccination.

It is likely that the persistence of the IrrL3 vaccine in combination with the Ribi adjuvant resulted in the prolonged antibody production. The longevity of IrrL3 is unknown, however, these larvae have not been found in the arteries at times when challenge larvae are being recovered. Nonirradiated larvae penetrate the intestinal arteries by D7, a time that correlates with the molt to the L4 stage. It is not known if the IrrL3 are capable of molting to the L4 stage and/or lack the necessary enzymes needed for arterial penetration and migration. Regardless of the case, it is hypothesized that IrrL3 are killed within the intestinal submucosa within the first week following administration. Therefore, the combination of Ribi-SAWA and IrrL3 likely resulted in prolonged antigen presentation to B-cells within follicular regions and enhanced stimulation by T helper cells and cytokines.

Previous studies reported that ponies protected against *S. vulgaris* through vaccination with IrrL3 had elevated total IgG antibodies to the L3 surface. It was further shown that this response was reduced in nonprotected Ribi-SAWA vaccinates (Monahan et al., 1994). Due do these differences in L3 surface antibody responses between protected and nonprotected ponies, it was hypothesized that the protected animals may display a dominant IgG subisotype response to the L3 surface. Data from this study, however, showed that ponies protected against *S. vulgaris* (R+I+C and I+C) had elevated levels of all IgG subisotypes to both early and developing L3 surface

antigens. Moreover, the levels of L3-specific IgGa, IgGb and IgG(T) were elevated earlier in protected animals (D 9) compared to nonprotected R+C animals (D 14). As well, nonvaccinated ponies lacked a demonstratable L3 surface-specific IgG antibody response until D 28. Similar to the soluble larval antibody responses, the ponies vaccinated only with IrrL3 demonstrated a trend towards increased IgGa and IgG(T) to developing L3 compared to animals in the other treatment groups on D 14. The IgGb titers in all animals were lower compared to IgGa and IgG(T) titers particularly on D 14 reaching only 1:1000 levels. The increased L3 surface antibody response in R+C ponies on D 14 was likely due to cross-reacting antibodies to SAWA induced by this vaccination protocol. It has been demonstrated through absorption studies using rabbit anti-adult worm sera that cross-reacting antibodies exist between L3 surface antigens and adult soluble somatic antigens (Klei et al., 1983). Although L3 surface antibodies were present in R+C ponies, these animals were clearly not protected from the challenge infection. The presence of these antibodies in nonprotected R+C animals does not rule out a role for an IgG ADCC-like protective immune mechanism. It is possible that the R+C ponies simply did not have the necessary antibodies and eosinophils present at a sufficient time for parasite elimination. As reported in Chapter 3, there was a delayed and diminished peripheral eosinophilia in nonprotected (R+C and N+C) ponies and a reduction in the number of cecal eosinophils on D 28. In contrast, both peripheral and cecal eosinophils were increased in ponies that were protected against the challenge infection (R+I+C and I+C). Having both L3 surface specific antibodies and eosinophils present during the first 9 days of infection may be required

for L3 killing. The specific IgG subisotypes, however, that bind equine eosinophils have not been reported.

Antibody-dependent cell-mediated cytotoxicity of migrating larvae has been hypothesized to be a major effector mechanism in a number of helminth infections including *A. cantonensis* (Yoshimura et al., 1983), *S. stercoralis* (Nolan et al., 1995) and *T. spiralis* (Venturiello et al., 1995). Evidence of ADCC-like responses has been demonstrated with *S. vulgaris*. In vitro analysis has shown that eosinophils and neutrophils from *S. vulgaris* infected ponies express more Fc and complement receptors than do eosinophils and neutrophils from strongyle naïve ponies (Dennis et al., 1988). In addition, it has been demonstrated that activated eosinophils from *S. vulgaris* infected ponies adhere to and immobilize L3 in the presence of immune serum or immunoglobulins (Klei et al., 1992).

In summary, these data indicate that antibodies of multiple isotype and IgG subisotype are generated to SAWA, L4 and L3 soluble antigens and to L3 surface antigens in ponies protected against *S. vulgaris*. The outer cuticular surface of nematodes and excretory/secretory proteins typically represent the main immunogenic challenge to an infected host (Maizels and Selkirk, 1988). Unique *S. vulgaris* larval stage and surface proteins are recognized by immune serum (Klei et al., 1983; Monahan et al, 1994) and demonstrate that there are shifts in protein expression during the development of *S. vulgaris*. The relationship of these proteins with immunity is currently unknown, however, they may represent crucial targets in determining whether successful parasitism is established. Considerable experimental evidence also exists in other nematodes that the carbohydrate component of nematode antigens is important for

antibody recognition (Dell et al., 2001). The binding specificities of equine IgG isotypes are currently unknown. However, an overall increase in soluble and surface IgG antibodies in conjunction with elevated eosinophils correlated with protective immunity to *S. vulgaris*. An early IgG subisotype response to the surface of both early and developing larvae was demonstrated in protected animals compared to nonprotected animals. Furthermore, all IgG subisotypes recognized both early and developing *S. vulgaris* L3 and therefore, it is unknown if one specific IgG subisotype is essential for protection. The correlation between these increased antibodies and eosinophils continue to support the hypothesis that ADCC-like mechanisms are important for protection against *S. vulgaris* in equids.

SUMMARY

The purpose of this dissertation was to further characterize the equine T-cell and humoral immune response to nematode parasites and to address mechanisms by which these responses may be regulated. Experiments were developed to study cytokine, antibody and cellular immune responses in ponies with either a mixed gastrointestinal parasite infection or those infected with the single nematode, *Strongylus vulgaris*. The initial experiment described in Chapter 2 was designed to determine the effect of varying levels of gastrointestinal parasite burdens on the immune response to subsequent heterologous protein immunization. The hypothesis was that the immune response to heterologous vaccination would improve when the ponies' parasite burdens were reduced and that parasitism would induce a biased Th2 cytokine response to KLH immunization. The experiments described in Chapters 3 and 4 were designed to study the specific regulation of equine T-cell and humoral immune responses and therefore the well-defined *S. vulgaris* helminth-naïve pony model was employed. In Chapter 3, the hypothesis was that a milieu of Th1 cytokines would down-regulate Th2 cytokine induction and IrrL3 vaccinated ponies would be susceptible to *S. vulgaris* challenge. Building on this premise, the hypothesis in Chapter 4 was that protected ponies would elicit a distinct anti- *S. vulgaris* antibody profile that would be altered by a Th1 cytokine milieu.

Although several studies have been published on the effects of parasitism on heterologous vaccination in rodent models, Chapter 2 details the first experiment of this type in equids. Mixed breed, naturally infected ponies, were randomly allocated to one of three treatment groups based on fecal strongyle egg counts. The treatment groups

were: 1. moxidectin 2% oral gel (400 μg kg^{-1}) given once; 2. pyrantel pamoate (6.6 mg kg^{-1}) given twice at a three-week interval and 3. non-treated controls. All ponies were immunized with a single intramuscular injection of 2 mg KLH. Serum samples were collected for antibody analysis and PBMC were collected for lymphoproliferation assays and cytokine mRNA quantification.

Cyathostomes comprised the largest population of parasites recovered. The treatment protocol was successful in establishing ponies with a range of parasite numbers, which were designated as high (not treated), medium (pyrantel pamoate), and low (moxidectin). The level of parasitism did not significantly affect the ability of PBMCs to proliferate in response to protein (KLH) and mitogen stimulation. However, there was a trend toward an improved lymphoproliferative response in ponies with the least number of parasites, especially in comparison to pre-vaccine levels. The PBMC from the lightly parasitised animals also produced significantly higher levels of IL-4 and displayed a trend towards increased INF-γ production following KLH stimulation. There were no differences, however, in IL-5 or IL-2 production among ponies with varying levels of parasitism. Ponies with light parasite burdens also demonstrated a trend toward increased levels of KLH-specific total immunoglobulin, IgG(T) and IgA compared to heavy and moderately parasitised animals.

In Chapter 3, ponies were sensitized with a Th1 cytokine-inducing combination of Ribi adjuvant and soluble adult worm antigen (Ribi-SAWA) prior to and concurrently with the Th2 cytokine-inducing IrrL3 immunization. The goal of this immunization regime was to investigate the potential cross regulation of Th1 cytokines on the induction of Th2 cytokines in equids. It was further used to demonstrate the

importance of the Th2 cytokine response in protective immunity to *S. vulgaris*. In this experiment, yearling parasite-free ponies were allocated to one of five treatment groups. Ponies in the first treatment group were vaccinated intramuscularly with Ribi-SAWA three weeks prior to the concurrent immunization with Ribi-SAWA and orally administered IrrL3. A second IrrL3 vaccine and third Ribi-SAWA vaccine were administered after an additional three weeks. On day 0 (D 0) these ponies were orally challenged with 1000 non-irradiated *S. vulgaris* L3 and were designated as R+I+C. The remaining four treatment groups served as controls and were as follows: (I+C) IrrL3 given twice at a three-week interval and challenged; (R+C) Ribi + SAWA given three times at a three-week interval and challenged; (N+C) nonvaccinated and challenged; (N) nonvaccinated and nonchallenged ponies. Pony health was monitored daily and blood samples were collected weekly for CBC analysis. On D –5, D 9, and D 28 CLNC and PBMC were collected for cytokine mRNA quantification. Larvae were recovered at necropsy (D 28) and cecum, liver and ileo-cecal-colic artery tissue sections were collected for histology.

During the pre-challenge period, R+I+C ponies exhibited a more severe clinical response to vaccination. Compared to the ponies in the other treatment groups, these animals were pyrexic and depressed for more days following the first and second IrrL3 vaccination. Following challenge, however, all IrrL3 vaccinates (R+I+C and I+C) displayed a trend toward fewer episodes of depression, pyrexia, anorexia, colic and were protected from larval migration. The mesenteric arteries of the protected ponies (R+I+C and I+C) also had fewer gross changes and histologically they retained normal arterial architecture. In contrast, nonprotected ponies (R+C and N+C) displayed gross

and histologic lesions typical of verminous arteritis. Ponies in the R+I+C group had higher average hepatic lesion scores than the ponies in the other four treatment groups. These lesions included severe expansive areas of periportal inflammation, fibrosis, and biliary hyperplasia. The inflammatory infiltrate was composed predominately of degranulated eosinophils. Although I+C ponies also displayed an eosinophilic periportal fibrosis, the response was markedly less severe compared to R+I+C ponies. These lesions were minimal or nonexistent in the other three treatment groups.

IrrL3 vaccinates (R+I+C and I+C) developed an anamnestic eosinophilia following *S. vulgaris* challenge. The response, however, in R+I+C ponies was reduced and delayed compared to I+C animals. While there were no differences in the number of cecal eosinophils between protected ponies, vaccination with Ribi-SAWA only (R+C) suppressed the number of submucosal and lamina propria eosinophils. There were no significant differences in cecal mast cell numbers among the five treatment groups.

Cytokine copy units of IL-4, IL-5, IL-13 and INF-γ in CLNC and PBMC were quantified by RT-PCR. The CLNC IL-4 mRNA levels increased following challenge in all ponies receiving larvae (R+I+C, I+C, R+C, and N+C). Correlating with blood eosinophil levels, the I+C and to a lesser degree the R+I+C ponies demonstrated a trend toward increased IL-5 on D -5 and D 9 in the CLNC. Whereas all challenged ponies had elevated IL-13 levels on D 9, the protected ponies (R+I+C and I+C) had significantly greater IL-13 levels than ponies in the other three treatment groups. The INF-γ mRNA production was not elevated in any treatment group until D 28 and R+I+C ponies were the only group with significantly increased levels. Further analysis of the

CLNC INF-γ response revealed that two of the four ponies in the R+C group produced 40-fold more INF-γ copy units and 18-fold fewer IL-4 copy units on D 9 compared to the other 2 ponies in this treatment group. Ponies that exhibited the high INF-γ response had more than twice as many larvae (100 verses 41), exhibited a more severe clinical response to challenge infection and demonstrated a trend toward higher ICC arterial lesion scores.

Analysis of PBMC cytokine mRNA levels revealed that IL-4 levels in the R+I+C were elevated on D 9 and their values were significantly greater than the R+C, N+C and N pony groups. The PBMC IL-5 levels were not elevated in any treatment group until D 28 when the I+C ponies displayed an increased response. The PBMC IL-13 levels were low in all treatment groups before and after challenge. The R+C ponies had a trend toward increased PBMC INF-γ levels on D -5 that declined following administration of the challenge infection.

In Chapter 4, serum from ponies used for experiments described in Chapter 3 was used to further characterize the protective equine antibody response to adult and larval *S. vulgaris* antigens. In addition, an attempt was made to correlate cytokine responses with antibody isotype and IgG subisotype production. Levels of total IgG, IgGa, IgGb, IgG(T) and IgA to SAWA, L4 and L3 antigens were determined by ELISA using sera collected on days 0, 3, 9, 14, and 28. Total IgG levels to all soluble antigens in R+I+C ponies were consistently higher than those of R+C ponies at all times following challenge and both groups lacked a demonstrable anamnestic response. In contrast, the I+C ponies exhibited an anamnestic total IgG response to L4 and L3 antigens. The R+I+C, I+C and R+C ponies showed detectable IgGa and IgA to SAWA

on D 0 that did not increase following challenge. However, in response to larval antigens (L4 and L3), the I+C and N+C ponies had increased IgGa and IgA responses on D 14 and D 28, respectively. The IgGb response in R+I+C ponies increased in an anamnestic fashion to SAWA. In comparison, I+C ponies had a trend toward reduced IgGb levels to all soluble worm antigens except to L3 antigens on D 28. The IgGb response to SAWA was the only antibody subisotype in R+I+C ponies that was shown to increase in an anamnestic fashion. The I+C, but not R+I+C ponies displayed an anamnestic IgG(T) response to all soluble worm antigens. The L3-specific IgG(T) response in R+I+C ponies however was elevated early in the challenge period compared to I+C animals. Similar to the other antibodies measured, IgG(T) was not elevated in N+C ponies until D 28. These increased IgG(T), IgGa and IgA responses to L4 and L3 in R+I+C and I+C animals correlated with protection.

The L3 surface specific total IgG was minimal in all treatment groups until D 9 when titers in protected animals (R+I+C and I+C) increased. The protected ponies also demonstrated a trend toward increased IgGa, IgGb, and IgG(T) to both early and developing L3 on D 9, whereas antibody levels in nonprotected ponies (R+C) were not elevated until D 14. In addition, the I+C animals appeared to have higher IgGa and IgG(T) titers to developing L3 on D 14. All the IgG subisotypes bound with equal intensity to early and developing *S. vulgaris* L3 and the predominance of one IgG subisotype was not observed.

CONCLUSIONS

Removal of internal parasites by effective anthelminthic treatment produced a trend toward improved KLH specific lymphoproliferative responses. In addition, lightly parasitised ponies also produced significantly higher mRNA levels of IL-4 in response to KLH stimulation and also displayed a trend towards increased INF-γ production. Although a biased Th1 or Th2 cytokine response was not demonstrated in these animals, these results suggest an improvement in overall T-cell responsiveness. Correlated with increased IL-4 secretion, ponies with low parasite also demonstrated a trend toward increased levels of KLH-specific total immunoglobulin, IgG(T) and IgA. These data suggest that heavy parasite burdens alter the cellular and humoral immune responses of ponies to protein immunization. Although the presence of the parasites did not polarize the immune response towards a Th2 phenotype, overall cellular and humoral immunity was improved when the parasites were removed.

Experiments performed in the *S. vulgaris* helminth-naïve pony model further support a central role for IL-5 and eosinophils in protection against *S. vulgaris*. Protected ponies (R+I+C and I+C) showed an early increase in CLNC IL-5 mRNA that corresponded with an anamnestic-like increase in blood eosinophils. The IL-5 response also appeared to be compartmentalized since the elevated levels in the CLNC were seen much earlier than in the PBMC. Administration of Ribi-SAWA reduced the CLNC IL-5 and blood eosinophil levels, however when IrrL3 were concurrently administered (R+I+C) the peripheral eosinophil response was partially restored and correlated with protection. In addition, protected animals (R+I+C and I+C) had similar levels of submucosal and lamina propria cecal eosinophils. Many of these eosinophils had

degranulated which was suggestive of activation and may have had an important role in larval killing. Future studies are directed toward elucidating the role of eosinophils and IL-5 in protection against *S. vulgaris* through the use of an anti-IL-5 monoclonal antibody.

The R+I+C animals had a greater infiltration of eosinophils and mononuclear cells within the hepatic periportal regions. Although I+C animals also demonstrated periportal inflammation, the response was not as extensive as that seen in the R+I+C ponies. Although the mechanisms responsible for this enhanced inflammatory response in R+I+C ponies are unknown this increased responsiveness may have resulted from elevated production of inflammatory mediators such as IL-1 or TNF-α from the dual immunization regime, or accelerated larval killing within the cecal submucosa due to increased antigenic exposure during vaccination.

A relationship between IL-4 levels, increased cecal mast cell numbers, and protection was not established in this model system. While IL-4 was elevated in both protected and nonprotected animals, the number of submucosal mast cells did not increase. In addition to mast cell proliferation, IL-4 has also been shown to be the key cytokine in the induction of IgE responses (Leonard, 1999). The production and role of IgE during immune expulsion of *S. vulgaris* is currently unknown since reagents are not available for measuring the equine IgE response. Although IL-4 is typically associated with expulsion of gastrointestinal parasites, there is not a consistent role for IL-4 in the elimination of tissue-migrating nematodes. Our findings suggest that IL-5 may be acting independently of the Th2 cytokine, IL-4, in providing protection against tissue-migrating stages of *S. vulgaris*. Nonetheless, elevated levels of the Th2 cytokine, IL-13,

were measured in the CLNC of protected (R+I+C and I+C) animals. These results indicate that both IL-13 and IL-5 may be important for immunity to *S. vulgaris*. Interleukin-13 is associated with protection against several gastrointestinal parasites in rodents and has also been shown to act independently of IL-4. The specific role for IL-13 in the *S. vulgaris* model is currently unknown.

Additional evidence was obtained that argues against a role for INF-γ in the protective immune response to *S. vulgaris*. Significant levels of INF-γ were not detected in the CLNC of either protected or nonprotected ponies until D 28. This late induction of INF-γ was most likely a result of inflammation and phagocytosis of dead larvae. Furthermore, the 2 ponies in the R+I+C group that experienced elevated CLNC INF-γ levels on day 9 had a higher larval recovery, worsened clinical response and more severe arterial lesions compared to the low INF-γ producers. It was hypothesized that Ribi-SAWA would elicit a predominate INF-γ response that would down-regulate production of Th2 cytokines and render ponies susceptible to *S. vulgaris*. Contrary to this hypothesis, animals that were immunized with both Ribi-SAWA and IrrL3 still produced a biased Th2 cytokine response and were protected against challenge. If the Ribi-SAWA did elicit an early Th1 response following the initial vaccination, the Th1 cytokines were subsequently down-regulated by the IrrL3 immunization. The persistence of the IrrL3 was likely a strong Th2 inducer that overcame the potential cross-regulation by the Th1-inducing Ribi adjuvant.

Analysis of the humoral immune response against *S. vulgaris* revealed that total IgG levels to SAWA, L4 and L3 soluble antigens were enhanced when ponies were immunized with both Ribi-SAWA and IrrL3. Furthermore, this vaccination regime

resulted in a prolonged antibody response in R+I+C ponies following immunization. Increased IgG(T), IgGa and IgA responses to L4 and L3 soluble antigens correlated with protection in R+I+C and I+C ponies. These antibody responses also corresponded with elevated Th2 cytokines (IL-4, IL-5, and IL-13) in these animals. Since IgGb was produced only in R+I+C ponies to SAWA, it is likely that this subisotype is not associated with protection. The relationship however between cytokines and antibody production are strictly corollary since the cytokine regulation of equine isotype and IgG subisotype induction is currently unknown.

An early (D 9) IgG response to the surface of both early and developing larvae was demonstrated only in protected (R+I+C and I+C) animals. Antibodies bound with similar intensity to both the early and developing *S. vulgaris* L3. All measured IgG subisotypes bound to the L3 surface of *S. vulgaris* and therefore, it was not clear if one specific IgG subisotype was sufficient for protection against *S. vulgaris*. The correlation between increased levels of antibodies against soluble larval (L4 and L3) and L3 surface antibodies with eosinophils in protected ponies continue to support the hypothesis that ADCC-like mechanisms are important for protection against *S. vulgaris* in equids.

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APPENDIX

Equine Veterinary Journal

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25 October 2001

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Dear Dr Klei

Altered immune responses to heterologous protein in ponies with heavy gastrointestinal parasite burdens

Further to our telephone/faxed correspondence of yesterday. I am now confirming by letter that we are happy for you to include the article "*Altered immune responses to a heterologous protein in ponies with heavy gastrointestinal parasite burdens*" as part of your doctoral dissertation. As you are aware this article is part of the forthcoming Immunology Special Issue of EVJ which has not yet been published and cannot therefore be reproduced in anyway other than for the specified purpose. Please make the appropriate acknowledgement to source.

Yours sincerely

Rachel Green

Altered immune responses to a heterologous protein in ponies with heavy gastrointestinal parasite burdens.

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Keywords: horse, heterologous immunization, nematode, immunity, vaccination, KLH

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VITA

Jenifer Dee Edmonds, the daughter of Edward and Nancy Johnson, was born in Caldwell, Idaho, on April 3, 1973. She attended elementary school and high school in Parma, Idaho, until graduation in 1991. While attending the University of Idaho in Moscow, Idaho, she pursued a degree in veterinary science and in 1994 was accepted to the Washington State University College of Veterinary Medicine in Pullman, Washington. After completing her first year of veterinary school she received her bachelors degree in veterinary science from the University of Idaho. Upon completion of the Doctor of Veterinary Medicine degree in 1998, she applied to the Louisiana State University School of Veterinary Medicine to pursue graduate training in veterinary parasitology. Her graduate work was guided by Dr. Thomas Klei, professor of parasitology. On June 3, 2000 she married Dr. Matthew Edmonds of Broken Arrow, Oklahoma, who recently had completed a doctoral degree at Louisiana State University School of Veterinary Medicine in veterinary microbiology.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Jenifer D. Edmonds

Major Field: Veterinary Medical Sciences

Title of Dissertation: T-Cell Cytokines and Equine Nematode Infections

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

October 18, 2001